PTHrP and calcium balance in gilthead sea bream,

Sparus auratus

PTHrP and calcium balance in gilthead sea bream, *Sparus auratus*

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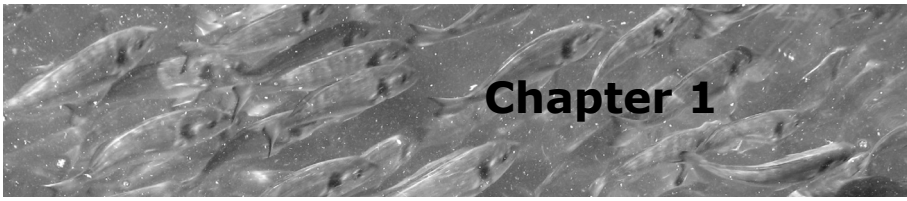

Chapter 1

General introduction

Wout Abbink and Gert Flik

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Calcium in fish

In the past three decades, numerous studies on fish calcium physiology (*e.g.* Wendelaar Bonga and Pang, 1991; Flik and Verbost, 1993; Riccardi, 1999), have established that in teleosts, as in terrestrial vertebrates, calcium plays a key role in a plethora of biochemical and physiological processes, which include vision, muscle contraction, vitellogenesis, signal transduction, blood coagulation and membrane permeability.

Fish have access to unlimited sources of calcium; the water and diet form the external, environmental sources of calcium and the skeleton and dermal scales, important for shape, armour, structure and muscle attachment, serve a role as internal reservoir for calcium as well. External calcium is taken up via the gills and the intestinal tract and the net flow of calcium is eventually determined by integumental (mainly branchial) efflux and intestinal excretion and by the control over these undirectional flows. In teleosts, about 99% of the total calcium pool is incorporated into the skeleton and dermal scales as calcium phosphates (brushite and apatite) and to a lesser extent as calcium carbonate (Flik et al., 1986).

In fish blood, calcium is either complexed (*e.g.* to citrate), protein-bound or present as free ion. The physiologically important free calcium fraction accounts for about half of the total calcium fraction in fish blood and is around 1.25-1.50 mmol l^{-1} (Hanssen et al., 1991). Fish regulate the ionic plasma calcium level more strictly than the protein-bound calcium level. This relates to the fact that even minor deviations from the set point ionic calcium concentration lead to severe stress and a disturbance of calcium balance and all calcium related physiological processes (Flik et al., 1995).

Unlike terrestrial vertebrates, which rely on their diet as calcium source, fish live in an environment with readily available calcium. Seawater has a calcium concentration of about 10 mmol l^{-1}, (fresh water contains about 0.1-2.0 mmol l^{-1} calcium) and the total plasma calcium concentration of marine fish ranges from 2-3 mmol l^{-1} (ionic fraction is around 1.50 mmol l^{-1}). Marine fish thus live in a hypercalcic environment and face a significant inward gradient of Ca^{2+}. One could argue that under such conditions regulation of calcium uptake is mainly a matter of control over influx of calcium from the water. Indeed, in fish, the hormone stanniocalcin plays a dominant role in calcium physiology as it exerts dominant anti-

hypercalcemic actions through control of calcium channel activity in the chloride cells of the gills and intestine (Wagner et al., 1998). However, as calcium availability from the environment varies and also hypocalcic conditions are encountered, for instance in estuarine and freshwater environments, one may anticipate that also hypercalcemic mechanisms must have been realised. Not only to antagonise stanniocalcin actions, but rather to regulate internal calcium stores associated with the bone compartments. Not surprisingly, fish possess a variety of calcium regulatory systems controlling integumental calcium handling, as well as bone compartments that appear to react rapidly to changes in environmental calcium availability and guarantee calcium balance (Wendelaar Bonga and Pang, 1991; Björnsson et al., 1999).

As indicated above, stanniocalcin (STC) is the dominant calciotropic hormone in fish (Verbost et al., 1993). STC is produced in the corpuscles of Stannius and is released in response to increased circulating calcium levels. STC acts as an anti-hypercalcemic hormone (Lafeber et al., 1988), since it inhibits the influx of calcium ions from water into the blood by closing Ca^{2+}channels in the apical membranes of gill epithelium cells (Perry and Flik, 1988).

Calcitonin (CT) is the second hormone in fish with hypocalcemic actions, although its role in fish physiology is less known than in mammals. Wagner et al. (1997) found that in rainbow trout (*Oncorhynchus mykiss*) CT acts as an inhibitor of branchial calcium transport and Foucheron-Peron et al. (1990) showed that circulating and branchial levels of CT vary during annual (reproductive) cycles, in accordance with cycling in calcium absorption activities. Interestingly, CT gene expression is not restricted to the main site of production, *viz.* the ultimobranchial glands (Pang, 1971), but was also found in the pituitary gland of Fugu (*Fugu rubipres*), with the most abundant expression in the proximal pars distalis, in cells that appear to secrete both growth hormone and CT (Clark et al., 2002).

Prolactin (PRL), growth hormone (GH) and somatolactin (SL) are hypercalcemic hormones and considered members of the 'prolactin gene family'; these pleiotropic hormones are nowadays appreciated as members of the large family of type I alpha-helical cytokines (Huising et al., 2006). These hormones are secreted by cells in the pituitary gland: PRL and GH by cells in the proximal pars distalis and SL by two populations of cells in the

pars intermedia (Weltzien et al., 2003). All three hormones are associated with osmoregulation and calcium regulation in fish. PRL has been studied most extensively with respect to calcium (Kaneko and Hirano, 1993; Mancera et al., 1993; Flik et al., 1994). PRL is activated in response to reduced environmental osmolality during adaptation to brackish- or fresh water of euryhaline teleosts. Reduced or increased external calcium levels stimulate, respectively inhibit PRL secretion and this points to hypercalcemic actions of PRL (Wendelaar Bonga et al., 1985). Indeed, several studies have substantiated hypercalcemic actions of PRL in fish (*e.g.* Flik et al., 1994; Santos et al., 1999).

SL was first described in fish in the pituitary gland by Rand-Weaver et al. (1991). Kakizawa et al. (1993) studied SL plasma levels and *sl* mRNA expression in rainbow trout (*O. mykiss*) and suggested a role for SL in calcium balance and demonstrated increased SL turnover at low calcium levels. Recently, two distinct sub-populations (SLα and SLβ) were described (Zhu et al., 2004). Although it is likely that these two populations have different functions, no studies have been done on this topic, but this item will be addressed in this thesis.

GH is a key hormone in the regulation of growth and development and is also involved in osmoregulation and reproduction (Kelly et al., 1999), processes that rely on a tightly regulated calcium balance (Flik et al., 1993). The role of GH on these processes is largely mediated by the production of insulin-like growth factor (IGF-I) in the liver (Sakamoto and Hirano, 1993).

Calcitriol -1,25$(OH)_2D_3$- is the active metabolite of vitamin D_3 (Sundell et al., 1993) and is a steroid hormone that exerts its effects through a high-affinity vitamin D_3-receptor (VDR; Lock et al., in press). Calcitriol stimulates intestinal calcium absorption (Swarup et al., 1991) and is a key factor in bone formation (Haga et al., 2004) in fish. The effects of calcitriol in fish are thus hypercalcemic, as they are in terrestrial vertebrates.

Cortisol is the main corticosteroid in fish, produced in the interrenal tissue (Wendelaar Bonga, 1997). Cortisol is the major stress hormone in fish, but its actions involve numerous physiological processes and extend to osmoregulation and calcium regulation (Flik and Perry, 1989). Cortisol has both glucocorticoid and mineralocorticoid actions (Wendelaar Bonga, 1997) and both mineralocorticoid and glucocorticoid type transcription factors have been found in fish (Bury et al., 2003). It remains to be established which

transcription routes are controlled by which receptors and even the possibility of additional steroid activity (next to that of cortisol), namely that of DOC, is at present a matter of debate.

Oestradiol-17β (E_2) stimulates vitellogenesis in fish, thereby influencing calcium balance (Guerreiro et al., 2002). The increase in calcium requirement for reproduction is achieved by mobilisation of calcium from the skeleton and scales and an increased calcium uptake from the water.

Parathyroid hormone-related protein (PTHrP), the hormone that takes a pivotal position in this thesis, acts as a hypercalcemic hormone in fish. In this introduction, a brief description is given of the discovery of PTHrP and the roles of the peptide in mammalian physiology. Next, the occurrence of PTHrP in the earliest vertebrates, sharks, skates and fishes, is reviewed and the calciotropic functions of PTHrP are addressed more specifically in fishes.

PTHrP is a hypercalcemic hormone in teleostean fishes, but also has para- and autocrine functions (Guerreiro et al., 2007). After the isolation and identification of fish PTHrP and PTHrP receptors and the subsequent development of recombinant protein and a real-time quantitative PCR, a calciotropic role of PTHrP in fish physiology could be assessed. PTHrP influences calcium physiology via regulation of calcium mobilisation from internal sources (bone and scales) and via calcium uptake from the environment (water and diet). Continuous variations in the need for calcium and in the availability of environmental calcium require fast calciotropes to guarantee calcium balance, in which PTHrP is pivotal for the fish. PTHrP is essential in fish bone physiology, *e.g.* in mineralisation and calcium reabsorption from the scales. Moreover, PTHrP plays a role in vitellogenesis, cortisol production, regulation of renal Mrp2 activity and melatonin synthesis. The plethora of functions of PTHrP in fish concern endocrine, paracrine and autocrine (and possibly intracrine) functions; calciotropic actions of PTHrP at the organismal and cellular level are prominent in fish. The strong conservation of the *pthrp* gene in the vertebrate lineage and the N-terminal similarity of the coded proteins relates to the important role of PTHrP in calcium physiology that is of paramount importance to all physiological processes. Recent and ongoing studies will contribute to our rapidly expanding knowledge of the original physiological functions of

PTHrP in teleost fish. Below we will deal with various aspects of PTHrP into more detail.

PTHrP, its discovery

PTHrP was discovered in 1987 in humans as the circulating peptide responsible for the syndrome of humoral hypercalcemia of malignancy (HHM; Moseley et al., 1987). PTHrP and parathyroid hormone (PTH), the main hypercalcemic hormone in vertebrates, evolved from a common, ancestral gene. Within a species, the two proteins share a high, about 70% N-terminal amino acid (aa) homology and bind with similar affinity to a shared PTH/PTHrP receptor (Jüppner et al., 1991). PTH is secreted from the parathyroid glands; however, *pthrp* is expressed in and PTHrP secreted by a variety of tissues, including many epithelia (de Papp and Stewart, 1993). In humans, there are three different *pthrp* mRNA transcripts, encoding for isoforms of 139, 141 and 173 aa long (Mangin et al., 1989). These isoforms have different physiological functions; have three bioactive subdomains (N-terminal, mid segment and C-terminal sequences) and mostly act in an intra- auto- or paracrine fashion. Besides the pathological effects of overproduction of PTHrP in HHM, the main functions of PTHrP in 'normal physiology' are regulation of transepithelial calcium transport, regulation of smooth muscle tonus (relaxation of stomach, urinary bladder and arterial segments), tissue growth and differentiation, and cell proliferation (de Papp and Stewart, 1993; Philbrick et al., 1996; Martin et al., 1997). The amino acid sequence of PTHrP is strongly conserved among mammalian species, which indicates its importance in mammalian physiology; the presence of PTHrP in fishes (and elasmobranch species) suggests that this signal protein has played an important role in vertebrate physiology throughout evolution.

PTHrP in the earliest vertebrates

PTHrP has been detected in tissues and plasma of the lamprey (*Geotria australis*; Trivett et al., 2005), in sharks and rays as well as in a lungfish (*Neoceratodus forsteri*; Danks et al., 1998), establishing that this protein is at the roots of vertebrate evolution. Elasmobranch species have a cartilaginous skeleton that contains calcium minerals, albeit less compared than in bony

fishes, which have a skeleton with apatite as the main mineral, just as mammals. PTHrP immunocross-reactivity was found in tissues of the dogfish (a shark), *Scyliorhinus canicula* (Ingleton et al., 1995) and in the red stingray (*Dasyatis akajei*), a PTH/PTHrP receptor (now called PTH1R) was demonstrated (Akino et al., 1998). Trivett and colleagues (2002) detected PTHrP in tissues of representative species from different elasmobranch families, using antisera against human (1-14)PTHrP, (1-16)PTHrP and (67-84)PTHrP. In general, the distribution of PTHrP in elasmobranch species is similar to that in mammals, pointing to the conservation of PTHrP and PTHrP production sites in evolution. Basal, circulating PTHrP levels in elasmobranch species (Ingleton et al., 1995; Akino et al., 1998; Trivett et al., 1999, 2002) are consistently higher than the PTHrP levels found in healthy humans and similar to the levels found in patients with HHM. Caution in the outcome of the studies on elasmobranches is needed, because in all instances heterologous antisera were used and specific data with homologous probes are necessary to make the observations unequivocal.

PTHrP in teleosts

In the late 1970s, the research on PTH-related factors in teleosts started. The development of specific antisera for immunohistochemical studies and quantitative immunoassays and the development of molecular biological techniques have clearly advanced the evidence for the presence of PTHrP proper in teleosts. The first reports on a PTH-like substance in teleostean species gave evidence for a hypercalcemic factor in the fish pituitary gland that was shown to be immunologically related to mammalian PTH (Parsons et al., 1978). Next, in extracts of corpuscles of Stannius from European eel (A*nguilla anguilla*), a PTH-like substance was demonstrated by immunocytochemistry; surprisingly, this substance exerted hypocalcemic actions in fish, a phenomenon that remains enigmatic (Milet et al., 1982; Lopez et al., 1984). However, it was confirmed by the demonstration of similar hypocalcemic effects of bovine (1-34)PTH and corpuscles of Stannius extracts (Wendelaar Bonga et al., 1986) injected into fish adapted to low calcium water. Differences in tissue sources (different species), extraction procedures and bioassays used may be at the basis of the discrepancies in these studies. The corpuscles of Stannius are not homogeneous in cell make-

up (at least two cell types; Wendelaar Bonga et al., 1989) and products produced, and it may thus be that differential effects of PTHrP and stanniocalcin in an extract (and dependent on the relative abundance of these products) have caused these apparent discrepancies. Harvey et al. (1987) detected an immunoreactive PTH-like substance in the circulation and in several tissues of trout (*Salmo gairdneri*) and goldfish (*Carassius auratus*). Kaneko and Pang (1987) demonstrated the presence of a PTH-like substance in brain of goldfish.

True immunoreactive PTHrP in teleosts was probably first shown in the pituitary gland of Coho salmon (*Oncorhynchus kisutch*) by Fraser and colleagues (1991), who suggested a role for the protein in calcium regulation and by Danks et al. (1993), who demonstrated PTHrP in the pituitary gland and plasma of sea bream (*Sparus auratus*) with an antiserum against human N-terminal (1-16)PTHrP. Molecular biology unequivocally demonstrated the presence of PTHrP in fish. The cloning of a *pthrp* cDNA from fugu (*Fugu rubripes)* revealed a 2.25kb cDNA gene product encoding for a 126 aa peptide (Power et al., 2000). Flanagan and co-workers (2000) cloned a 1.8 kb cDNA from sea bream, encoding a 125 aa gene that showed 85% overall homology with the fugu *pthrp* gene. Rotllant and Du (2004; accession number AY608915.1) and Rubin et al. (2005; accession number DQ022615.1 and accession number DQ022616.1) reported the presence of the zebrafish *(Danio rerio) pthrp* gene which establishes its wide occurrence in fishes. As fugu and zebrafish diverted 300 million years ago (Hedges, 2002), we may conclude that the gene is very well conserved indeed.

The piscine PTHrP shares an overall 36% aa homology with its tetrapod counterpart. However, this homology varies greatly per gene region: indeed, the N-terminus shares around a 62% homology with mammalian PTHrP. The piscine PTHrP lacks the 'mammalian' C-terminal domain responsible for osteoclast inhibitory activity, which suggests that this function arose after the water-land transition of vertebrates. Also, the piscine *pthrp* gene, encoding for a protein with insertions between positions 38 and 54 (fugu) or 38 and 65 (sea bream) that is absent in the mammalian *pthrp* gene and this opens the possibility of a unique function for this segment of the peptide in fish physiology (Power et al., 2000).

PTHrP was quantified in plasma by radioimmunoassay using heterologous antisera raised against human peptide (Danks et al., 1993;

Devlin et al., 1996) and later with homologous antisera (Rotllant et al., 2003; Abbink et al., 2004, 2006). As holds for elasmobranch species, PTHrP levels in teleostean plasma are consistently and significantly higher than these values for humans (basal circulating levels of around 0.5-2.5 pmol l^{-1} in humans and 0.1-0.6 nmol l^{-1} in fish). The difference in levels of circulating PTHrP in elasmobranches and fishes compared to mammals may indicate that PTHrP has lost its endocrine function in normal physiology of the latter and has assumed more local and paracrine functions.

In situ hybridisation (Danks et al., 1998; Trivett et al., 1999; Ingleton et al., 2002) and immunohistochemistry (Devlin et al., 1996; Trivett et al., 1999; Flanagan et al., 2000) have demonstrated the *pthrp* mRNA and PTHrP protein in gills, operculum, kidney, pituitary gland, brain, saccus vasculosus, muscle, skin, spleen, liver and intestine. This widespread distribution of the *pthrp* gene and protein reflects the wide range of functions that are reported for PTHrP in fish and suggests endocrine, paracrine, autocrine and intracrine functions; to date, no reports on intracrine effects in fish tissues are available. The (high) circulating PTHrP-levels in the range of other protein endocrines (nmol l^{-1}) would be in line with a classical endocrine function for (pituitary) PTHrP (Danks et al., 1993) as is its presence in pituitary cells (Ingleton et al, 1998; Abbink et al., 2006).

PTHrP was demonstrated in periodic acid Schiff (PAS)-positive cells identified as a sub-population of somatolactin (SL)-producing cells (Abbink et al., 2006). Interestingly, in the sea bream pituitary gland, PTHrP is found only in the SL-producing cells that would be similar to the recently described SLα cells in zebrafish (Zhu et al., 2004). Indeed, in an earlier study, Ingleton et al. (1998) reported that in sea bream, PTHrP and SL are both located in PAS-positive cells and that some cells do contain both PTHrP and SL. SL is a hormone from the prolactin gene family and is structurally related to both PRL and growth hormone. SL may play a role in regulation of the calcium balance of the fish: changes in SL plasma levels and *sl* pituitary gland mRNA expression at low ambient calcium were observed, albeit only after several days (Kakizawa et al., 1993). This makes short-term effects of SL on calcium balance unlikely. However, the presence of PTHrP in SLα cells could indicate that a correlation between somatolactin cell activity and fast hypercalcemic effects reflects a SLα cell activity mediated through PTHrP.

Two other sites in fish may contribute to circulating PTHrP levels, *viz.* the gills and the corpuscles of Stannius (see discussion above). The latter glands can be easily removed in certain fish (*e.g.* stanniectomy of eel), and such an experiment is strongly indicated, but awaits further development of immunoassays to detect PTHrP in these species. The gills were suggested to be evolutionary related to the parathyroid gland in vertebrates (Okabe and Graham, 2004), but to us the gills seem a less likely site for circulating PTHrP, as we see lower or unchanged PTHrP levels in fish that have up-regulated levels of *pthrp* mRNA when confronted with limited access to calcium in water and/or diet or when made vitamin D-deficient (Abbink et al., 2006; Abbink et al., 2007).

PTH1R, the most common receptor in teleosts that binds PTHrP, is a G-protein-coupled receptor with similar affinities for PTHrP and PTH (Gensure et al., 2005). Three PTH receptors have been cloned in zebrafish (*Danio rerio*), which are referred to as PTH1R, PTH2R and PTH3R (Rubin and Jüppner, 1999). The PTH2R is activated by human PTH, but not by the human or teleost PTHrP, which interestingly would suggest the presence of PTH in fish (Rubin et al., 1999). Indeed, Danks and colleagues (2003) identified a gene encoding for an 80 aa PTH in fugu and the predicted protein N-terminus of fugu PTH is homologous to the N-terminus of tetrapod PTH. However, the C-termini of fugu PTH and tetrapod PTH show no homology at all. Apparently, there is a lower evolutionary pressure on the fish *pth* gene than on the fish *pthrp* gene, which shows a significant degree of C-terminus homology with human PTHrP. In the zebrafish genome, two *pth* genes were found (*pth1* and *pth2*) that are highly homologous to the human *pth* gene and the proteins derived from the genes have high affinity for the PTH1R (Gensure et al., 2004). Also in pufferfishes (*Takifugu rubripes* and *Tetraodon fluviatilis*) two PTH hormones (PTH_A and PTH_B), two PTHrPs ($PTHrP_A$ and a novel $PTHrP_B$) and a PTH-like ligand (PTH-L) were identified; PTH-L has both PTH and PTHrP characteristics and *pth-l* is proposed to represent the ancestor of the *pth*/*pthrp* gene (Canario et al., 2006).

Our knowledge of the physiological functions of PTHrP in fish is recently rapidly expanding. The availability of recombinant sea bream PTHrP allowed the development of region-specific detection and

quantification to define post-translational and postsecretory processing of the protein and to assess its bioactivities in fish (Anjos et al., 2005). A real-time quantitative PCR was designed by Hang et al. (2005) for sea bream tissues to measure mRNA expression levels for *pthrp*, *pth1r* and the calcium sensing receptor *casr*, which, in humans, has a set point that can detect minor changes (as small as 0.2 mmol l^{-1}) in blood Ca^{2+} and regulates secretion of calcemic endocrines, including PTHrP, to strictly control the plasma Ca^{2+} levels (Chattopadhyay et al., 2000). Quantitative analysis of these messenger RNAs proved to be a powerful tool to study the role of PTHrP in fish calcium handling (Abbink et al., 2006).

Functions of PTHrP in fish

The structure of the *pthrp* gene and the presence of the protein and its receptors in a wide array of tissues are under intensive study. A strong focus is on the function of PTHrP in teleosts that relates to calcium physiology. An important line of research addresses how PTHrP influences calcium physiology via mobilisation of calcium from internal bone compartments (skeleton and dermal scales) and via calcium uptake from the environment (water and/or diet; external calcium sources). These two different faculties of calcium mobilisation for calcium balance imply the presence of different and possibly independent PTHrP systems for calcium regulation in fish.

Calcium balance

N-terminal (1-38)PTHrP enhances in a concentration-dependent way the accumulation of calcium in larval sea bream (Guerreiro et al., 2001). The larval undirectional calcium influx (Ca^{2+}-uptake from the water via chloride cells) is stimulated, whereas the epithelial efflux is reduced or unaffected; through these combined effects a strong positive net uptake of calcium ions from the environment into the fast growing larvae occurs, supporting the rapid growth of the skeleton. Little is known about presumed effects on early development of fishes, but clearly this calciotropic action is of major importance in normal skeletal growth. Remarkably, the increase in calcium uptake from the water via the integumental chloride cells is accompanied by a 30% reduction in the drinking rate of the fish, which could be a compensatory mechanism to avoid excessive calcium load from the seawater

drunk. Clearly, the findings indicate that PTHrP causes a shift in the way calcium is taken up by the fish and that it orchestrates multiple sites for calcium handling, via gills/skin, intestine and likely kidney (see below).

Indeed, a recent study by Fuentes et al. (2006) established a physiological role for PTHrP in intestinal calcium regulation. Increased intestinal calcium uptake was measured after 30 minutes of exposure to 6 or 30 nmol l^{-1} (1-34)PTHrP *in vitro*, in all three regions of the intestine tested in this study (duodenum, hindgut and rectum). In combination with a remaining (duodenum, hindgut) or decreasing (rectum) intestinal calcium efflux, the overall observation was an up to 4-fold increased net calcium accumulation, depending on the region of the intestinal track. The regional differences that were observed indicate that the calcium transport mechanisms involved vary between the regions, possibly by different active signalling pathways or by the involvement of different receptors (in addition to the PTH1R). The results clearly show that the intestinal tract is (in addition to the gills) a key target for PTHrP dependent calcemic control.

When PTHrP stimulates calcium uptake from the environment, decreases in environmental calcium availability and an imminent hypocalcemia should provoke enhanced reaction PTHrP-signalling as may predicted for any feedback control mechanism. We assessed the PTHrP system in juvenile sea bream confronted with calcium constraint in water and diet (Abbink et al., 2004, 2006). In gill tissue, expression levels of the *pthrp* and *pth1r* genes were up-regulated on the longer term after calcium constraint; short-term (4 hours) constraint did not provoke changes. However, in the pituitary gland, both short- and long-term exposure to calcium constraint resulted in an unexpected down-regulation of *pthrp* and *pth1r* expressions. These observations point to two different and possibly independent responses of PTHrP systems. The pituitary gland PTHrP system is rapidly and persistently down-regulated at the level of mRNA production. Plasma PTHrP levels are not significantly affected, which we take to indicate that the lower calcium turnover under environmental calcium constraint results in lower PTHrP turnover as well. Direct demonstrations of changes in PTHrP turnover await further experimentation. The up-regulation of the branchial PTHrP system during calcium constraint correlates well with a long-term adjustment of uptake mechanisms. The precise role of PTHrP in calcium acquisition remains to be

elucidated: branchial and intestinal influxes are very much lower under calcium constraint; yet net calcium accumulation is hardly affected (Fig. 1). Clearly then, PTHrP must limit calcium loss via branchial, intestinal and renal pathways.

The physiologically most relevant fraction of calcium in the blood is the ionic fraction, which is most strictly regulated. Calcemic endocrines react swiftly to changes in external calcium availability or increased physiological needs for calcium (rapid growth, vitellogenesis; Björnsson et al., 1999). As indicated above, circulating PTHrP levels remain rather constant or show only mild changes in response to a low water calcium level. Surprisingly, a mildly positive correlation between plasma ionic calcium and plasma PTHrP was recently established (Fig. 2; Abbink et al., 2006), which we tentatively assume to reflect lower PTHrP turnover at increased plasma calcium levels; also, this would support an endocrine calcium-regulatory function for PTHrP. This is corroborated and strengthened by a similar positive correlation between plasma ionic calcium and pituitary gland *pthrp* mRNA content.

Skeletal and scale physiology

The skeleton and dermal scales represent significant reservoirs of calcium, with in some fish about 99% of the total calcium pool being incorporated mainly in the form of calcium phosphates (Flik et al., 1986). Plasma PTHrP levels increase with increasing body weight of fish and mildly positive and strong correlations between plasma PTHrP and the whole body content of the main minerals in bone (calcium, phosphorus and magnesium) strongly suggest that PTHrP is involved in skeletal calcium physiology (Abbink et al., 2007). A research for a role of the bone compartments in metabolic clearance, production and secretion, as well as distribution space for PTHrP is strongly indicated by these studies. Somehow, plasma PTHrP levels in the fish reflect calcium, phosphorus and magnesium pool sizes in the fish. This could relate to a pool size-dependent, enhanced distribution space for PTHrP, or simply a requirement for enhanced PTHrP activity. Little is known about the role of PTHrP in fish mineral handling, but a short resume on PTHrP and phosphorus handling was published by Guerreiro et al. (2007).

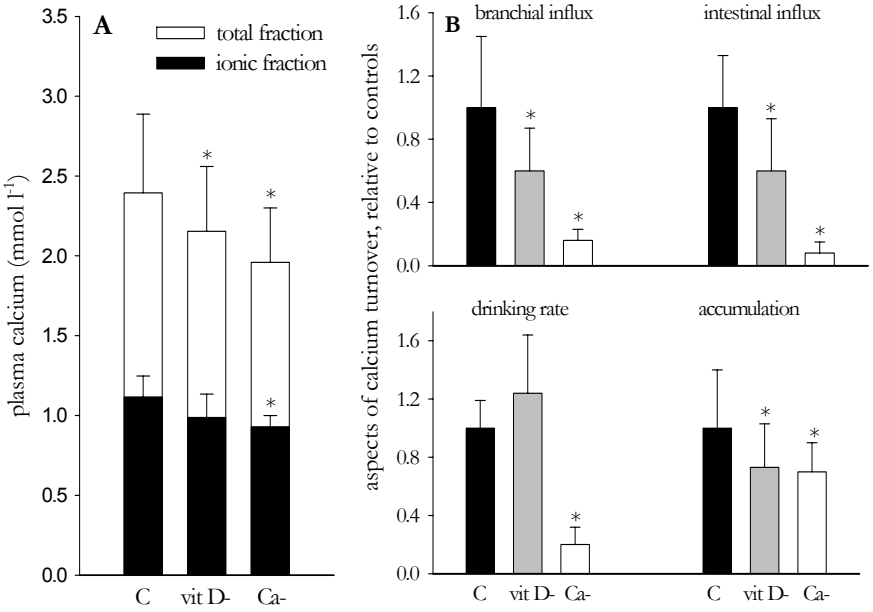

Figure 1. *Calcium balance of juvenile sea bream when confronted with either direct (via a calcium-deficient diet and strongly diluted sea water; Ca- group) or indirect (via a vitamin D-deficient diet; vit D- group) calcium constraint. Plasma calcium balance, best reflected by the important ionic fraction, remains at level to ensure long term survival of the fish (**Fig. 1A**). However, the calcium turnover is decreased (**Fig. 1B**): calcium influxes via gills and intestine, as well as drinking (not in vitamin D-deficient fish) decrease, resulting in a decreased calcium accumulation. Values are given as mean ± S.D. (Fig. 1A; n = 6-8). Values in Fig. 1B are given in nmol h^{-1} per g fish (fluxes and accumulation) and in nl h^{-1} per fish (drinking). Asterisks represent significant difference from the control group (group c; P<0.05).*

Calcium reabsorption from scales

Rotllant and co-workers (2005a) established an involvement of PTHrP in calcium reabsorption from scales, which was substantiated by the demonstration of the PTH1R in the tissue/cells associated with fish scales. Production of cyclic adenosine monophosphate (cAMP; to evaluate PTHrP induced signal transduction) and activity of tartrate-resistant acid phosphatase (TRAPC, a marker for osteoclastic activity in mammalian bone; the enzyme activity is also easily demonstrated in tissue associated with fish scales) were enhanced when treated with N-terminal (1-34)PTHrP. These

findings would suggest that N-terminal (1-34)PTHrP stimulates an osteoclastic activity in sea bream scales through the PTH1R via a cAMP/AC (adenylate cyclase) intracellular pathway.

Mineralisation

Calcitriol [1,25$(OH)_2$vitamin D_3], the active metabolite of vitamin D in vertebrates, plays a key role in bone formation (Haga et al., 2004) and stimulates intestinal calcium absorption (Swarup et al., 1991). Thus, calcitriol will contribute to a positive shift in calcium uptake and accumulation and its effects will be hypercalcemic. Calcitriol receptors were localised in several tissues involved in calcium handling (gill, intestine) in Atlantic cod (*Gadus morhua*; Sundell et al., 1992) and vitamin D metabolites, including calcitriol, have been found in plasma of various fish species (Horvli et al., 1998). Indeed, vitamin-D deficiency in juvenile sea bream impedes growth and slows down calcium turnover (*i.e.* calcium influx, efflux and accumulation rates decrease; Abbink et al., 2007). Changes in plasma PTHrP values and mRNA expression levels for the *pthrp* and *pth1r* genes in the pituitary gland and gill mark this first report of a correlation between the hypercalcemic factor PTHrP and calcitriol in fish (the correlation was earlier described in humans by Abe et al., 1998; Tovar Sepulveda and Falzon, 2003) and it was speculated that calcitriol-dependent bone formation is key in this correlation. An interesting parallel exists with the relationship between PTHrP and another steroid that affects calcium handling in fish, *viz.* estrogen. Estrogen is hypercalcemic in fish, but for this effect estrogen depends, at least partly, on a concerted action of PTHrP (Bevelander et al., 2006).

Osteonectin (OSN) plays a major role in bone mineralisation. OSN is described in (mammalian) skeletal biology as a calcium-binding glycoprotein that stimulates the mineralisation process following differentiation of the osteoblastic cell lineage (Estevao et al., 2005). High levels of ionic calcium up-regulate *osn* mRNA expression; in higher vertebrates, PTH is known to suppress *osn* gene expression (Nakajima et al., 2002). In agreement with this notion, treatment of sea bream scales in culture with 10 nmol l^{-1} (physiological) or 1000 nmol l^{-1} (pharmacological) (1-34)PTHrP abolishes *osn* mRNA expression; thus PTHrP-regulated bone mineralisation through regulation of the *osn* gene (Redruello et al., 2005) is

as old as the fishes (over 450 million years). The dermal scleroblasts that form the scales in teleostean fishes apparently harbour a VDR and PTHrP-receptors as well as osteoclastic and osteoblastic characteristics.

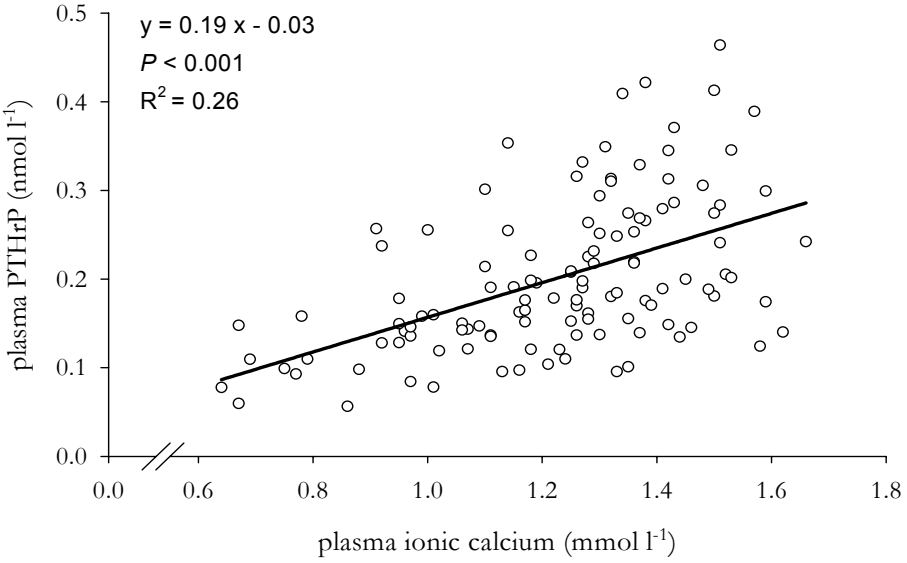

Figure 2. *The relationship between the plasma ionic calcium level and the plasma PTHrP level that was found, shows that PTHrP is involved in regulation of the calcium balance, which is best reflected by the plasma ionic calcium concentration.*

Other functions

Vitellogenesis

Vitellogenesis by the liver is triggered and maintained by estradiol-17β (E_2) and is accompanied by increased plasma calcium and phosphate levels. As described above, E_2-treatment increases whole body calcium accumulation and stimulates reabsorption of calcium from dermal scales (Guerreiro et al., 2002). As *pthrp* and *pth1r* are expressed in hepatocytes, a para-/autocrine role for PTHrP in vitellogenesis was indicated, in analogy to the situation in mammals (Cros et al., 1998). A direct involvement of PTHrP in fish vitellogenesis was recently shown: vitellogenin production in E_2-primed cultured sea bream hepatocytes is stimulated by homologous recombinant

PTHrP (Bevelander et al., 2006). E_2 stimulates the secretion of PTHrP into the blood stream preceding the rise in plasma calcium levels. This makes PTHrP the candidate of choice for the mediator of the hypercalcemic action of E_2 through mobilisation of Ca^{2+} from either external or internal sources.

Mrp2

Multidrug resistance protein 2 (Mrp2) is a carrier protein that is found among others in luminal membranes of renal proximal tubule cells, where it mediates the active secretion of (endogenous) waste products and xenobiotics (Schaub et al., 1997). In the killifish (*Fundulus heteroclitus*) nephron, Mrp2-mediated fluorescein-methotrexate transport (tubular secretion) is reduced by Ca^{2+}_i-dependent endothelin release via a PKC signalling pathway (Miller et al., 2002). Influx of Ca^{2+} (*e.g.* caused by toxicants) into the tubular cell through L-type calcium channels stimulates release of endothelin which results than in inhibition of Mrp2 activity. Also, extracellular high calcium levels reduce Mrp2 mediated transport. These observations prompted the question whether calcemic factors such as PTHrP, would interfere with this transport pathway. Indeed, PTHrP interferes with endothelin-regulated Mrp2-mediated transport (Wever et al., 2006). The inhibitory effect of recombinant PTHrP on Mrp2-mediated transport is concentration-dependent, with a maximal inhibition of 40% at 20-60 nmol l^{-1}, a concentration that indicates a paracrine action of PTHrP. The endothelin-induced inhibition is additive to the PTHrP-induced effect, indicating that the inhibitions proceed at least partly through separate intracellular pathways. Another interesting observation was that the endogenous PTHrP antagonist stanniocalcin (Verbost et al., 1993), which exerts actions via intracellular calcium and PKC pathways, reverses the combined PTHrP/ET inhibition of Mrp2-transport completely (Wever et al., 2006). Clearly, PTHrP has the nephron as target and studies on PTHrP effects on calcium and phosphate handling by fish kidney are warranted.

Cortisol

As in mammals (Nussdorfer et al., 2000), in fish the hypothalamus-pituitary-adrenal/interrenal (HPA/HPI) axis expresses *pthrp*, both in the pituitary gland and in the interrenal gland. In isolated and perfused interrenal tissue, sea bream (1-34)PTHrP rapidly stimulates the release of cortisol in a

concentration-dependent (range 10^{-6}–10^{-11} mol l^{-1}) way (Rotllant et al., 2005b). The EC_{50} of (1-34)PTHrP was 2.8 times higher than that of (1-39)ACTH and the increase in cortisol production in response to 10^{-8} mol l^{-1} (1-34)PTHrP was about 7-fold lower when compared with 10^{-8} mol l^{-1} (1-39)ACTH.

With the pars intermedia somatolactin cells as a main pituitary source of PTHrP and a regulatory role for pituitary PTHrP in stress-axis activity as suggested above, the search for the organisation of the pituitary vasculature or arrangement of extracellular fluid flows between pars intermedia and (rostral) pars distalis deserves further study.

In-vivo administration of physiological concentrations (1-34)PTHrP rapidly resulted in a dose-dependent inhibition of circulating cortisol (Guerreiro et al., 2006), although this effect was only short-lived (up to 5 h). Increased blood cortisol levels suppress the circulating PTHrP levels up to 24-fold, in line with a role for cortisol as a negative feedback regulator of PTHrP production (Guerreiro et al., 2006). The possibility of PTHrP-mediated corticotropic effects via (aspecific) activation of ACTH pathways was eliminated since the ACTH blocker corticotropin-inhibiting peptide (CIP) had no effect on (1-34)PTHrP-induced cortisol production. Furthermore, alignment of *acth* and *pthrp* sequences does not give any reason to suspect such interactions at the level of the respective receptors (PTH1R, PTH3R and MC2R). However, the expression of mRNA for *pth1r* and *pth3r* in interrenal cells suggests that the observed effect is mediated directly and specifically via PTHrP receptors, again indicating an auto- and paracrine action of PTHrP.

Melatonin

Melatonin is a product of tryptophan metabolism, mainly in the pineal gland and in the retina. It is synthesised in a rhythmic fashion, with high synthesis in darkness and low synthesis during the light phase of the light/dark cycle (Falcon, 1999). The production of melatonin is stimulated by high plasma Ca^{2+} levels (Begay et al., 1994) and inhibited following hypocalcemia (Meissl et al., 1996). In fish, melatonin is involved in development (Shi et al., 2004) and in the timing of parr-smolt transformation of Atlantic salmon (*Salmo salar*; Porter et al., 1998). In addition, melatonin suppresses osteoclast-specific TRACP activity, a protective mechanism against excess degradation of the scalar calcified matrix during vitellogenesis (Suzuki and Hattori,

2002). Together, these functional aspects, all (indirectly) related to calcium metabolism, raise questions as to a possible regulatory role of PTHrP in melatonin-steered physiology in fish. However, such an involvement has yet to be determined (Abbink et al., 2007).

Concluding remarks

After the isolation and identification of PTHrP and its receptors in teleosts, as well as its definitive detection in various tissues and in plasma, most recent studies have focussed on the calciotropic role(s) of PTHrP in fish physiology. The strong conservation of the *pthrp* (and *pth*) genes in the vertebrate lineage and the N-terminal similarity of the coded proteins relates to the key role of calcium physiology that is of paramount importance in all physiological processes. Life originated in a seawater environment and a strictly controlled calcium metabolism (calcium being toxic at elevated levels, inside the cell as well as outside the cell) is essential to all life in aqueous and marine environments.

Correlations between plasma ionic calcium and plasma PTHrP and between plasma PTHrP and pituitary gland *pthrp* mRNA expression show that PTHrP, as an endocrine factor, is important in maintenance of fish plasma calcium balance. Variation in the need for calcium (bone mineralisation, vitellogenesis) or the availability of environmental calcium (limited concentrations in water or diet) urge the endocrine system to respond rapidly to regulate the ionic calcium level. PTHrP-involvement in skeletal and scale physiology has been established in bone mineralisation (during growth and development) and in calcium reabsorption from the scales, when processes as vitellogenesis require extra calcium. The very strict control of calcium homeostasis (by a plethora of endocrines: PTHrP, PTH, PRL, stanniocalcin, calcitonin, E_2, calcitriol, cortisol, etc.) and plasma ionic calcium levels through swift endocrine adjustments make this a complicated field of research. However, a new array of paradigms with a key role for fish shows involvement of PTHrP in vitellogenesis, cortisol production, Mrp2 activity and melatonin synthesis. Such studies will rapidly expand our knowledge on this pleiotropic hormone. They show that the calciotropic actions of PTHrP concern organismal as well as cellular physiological phenomena. Studies on fish give insight in original functions of PTHrP-

regulated processes and, once again, show the power of comparative endocrinology.

Aim and outline of this thesis

The research presented in this thesis was carried out in the frame of the Research and Technological Development Project "Calcium, the backbone of fish culture: importance in skeletal formation, reproduction and normal physiology" (FISHCAL), with financial support from the Commission of the European Union, Quality of Life and Management of Living Resources specific RTD programme (Q5RS-2001-02904). The overall achievement of the project was to provide new information about calcium physiology in the euryhaline, marine teleostean gilthead sea bream (*Sparus auratus* L.) that was unavailable. FISHCAL aimed to identify causal factors in abnormal bone development in larvae, the mechanism by which calcium is incorporated into vitellogenin, the general requirements of calcium for normally growing fish and the function of PTHrP in these processes. The conclusions of the project were aimed to show the effects of diet and environment on calcium physiology of sea bream and provide guidelines for husbandry for sea bream to improve the quality of egg production, larval survival and control of pathological axial dystrophies and improvement in growth dynamics.

The research presented in this thesis covers aspects of the FISHCAL project aimed to elucidate hypercalcemic regulation in juvenile sea bream confronted with an experimentally induced limited access to environmental calcium. The principle to restrict sea bream in its access to calcium in water and diet was to activate hypercalcemic regulatory factors such as PTHrP and study the role of PTHrP in regulation of the calcium balance.

The experimental analyses addressed the regulation of calcium balance, measured as plasma calcium levels, unidirectional calcium fluxes, calcium accumulation, whole body pools and drinking rate and the role of PTHrP in these processes by analysis of circulating PTHrP levels and mRNA expression for *pthrp*, *pth1r* and *casr*, as well as the interaction between PTHrP and the calcitropic endocrines calcitriol and melatonin.

Chapter two describes the adaptive responses of juvenile sea bream in response to calcium limitation in water and diet in calcium regulation,

cortisol and PTHrP levels; we show that juvenile sea bream has an array of adaptive responses to deal with calcium constraint in its environment.

Chapter three focuses on the regulatory role of PTHrP as a fast acting hypercalcemic factor in response to environmental calcium constraint. Differential responses of *pthrp* and *pth1r* mRNA expression in gills and the pituitary gland indicate two separate and independently operating PTHrP systems, pointing to PTHrP as an endocrine, as well as a para- and autocrine factor in fish.

Chapter four concerns PTHrP actions under an indirect calcium constraint, realised by feeding the fish a vitamin D-deficient diet that results in a decreased plasma calcitriol level. The differential response of pituitary and branchial *pthrp* and *pth1r* expression is in accordance with the response to direct calcium constraint and further supports the presence of a peripheral brachial system and a central pituitary PTHrP system. We show that reduced *pthrp* and *pth1r* mRNA expression in the pituitary gland and lower PTHrP plasma values in vitamin D-deficient fish reflect lower turnover of PTHrP. Up-regulation of *pthrp* mRNA in gills indicates compensatory paracrine activity of PTHrP to guarantee well-regulated branchial calcium uptake. This is the first report to show a correlation between the hypercalcemic factors PTHrP and calcitriol in fish and we speculate that calcitriol- dependent bone formation is a key factor in this correlation.

Chapter five shows the effects of calcium constraint on melatonin production and discusses interactions of melatonin and the hypercalcemic factors PTHrP and calcitriol. We highlight the importance of Ca^{2+} in melatonin physiology and show that this hormone is related to the hypercalcemic factors PTHrP and calcitriol, suggesting that it is involved in the regulation of endocrine reactions responding to limited calcium availability.

Chapter six summarizes the results that were obtained in this thesis and discusses in further detail the main findings and future research possibilities.

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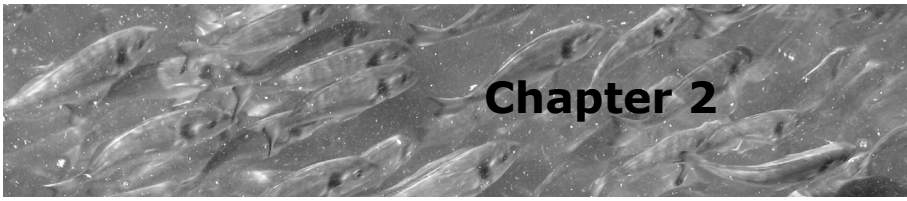

Chapter 2

Calcium handling in *Sparus auratus*: effects of water and dietary calcium levels on mineral composition, cortisol and PTHrP levels

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Abstract

Juvenile gilthead sea bream (Sparus auratus L.; 10-40 g body mass) were acclimatised in the laboratory to full strength (34‰) or dilute (2.5‰) seawater and fed normal, calcium-sufficient or calcium-deficient diet for nine weeks. Mean growth rate, whole body calcium and phosphorus content and accumulation rates were determined, as well as plasma levels of ionic and total calcium, cortisol and parathyroid hormone related protein (PTHrP; a hypercalcemic hormone in fish). When confronted with limited calcium access (low salinity and calcium-deficient diet) sea bream show growth arrest. Both plasma cortisol and PTHrP correlate positively with up-regulation of calcium uptake. Furthermore, a strong correlation was found between net calcium and phosphorus accumulation ($R^2 = 0.94$; $N = 16$; $P < 0.01$) and between body mass and whole body calcium and phosphorus content. Phosphorus accumulation is strongly calcium-dependent, as phosphorus accumulation decreases in parallel to calcium accumulation when the diet is calcium-deficient, but phosphorus-sufficient. We conclude that PTHrP and cortisol are involved in the regulation of the hydromineral balance of these fish, with growth-related calcium accumulation as an important target.

Introduction

In teleost fish, as in other vertebrates, calcium is of key importance for numerous physiological processes. The skeleton of vertebrates consists mainly of calcium phosphate and calcium carbonate. It serves an important role as it determines body shape, protective aspects (scales, bone plates) and as internal buffer for calcium and phosphorus. In teleosts, ~99% of the whole body calcium fraction is incorporated into bones and scales (Flik et al., 1986). Indeed, calcium is also of major importance for many other physiological processes, such as vision, muscle contraction, vitellogenesis, signal transduction, blood coagulation and membrane permeability (Riccardi, 1999).

In fish, blood plasma calcium is either complexed (*e.g.* to citrate), protein-bound or present as free ion. The free calcium fraction accounts for about half of the total calcium fraction and is the physiologically important fraction (Hanssen et al., 1991). Fish regulate their ionic plasma calcium level more strictly than their protein bound calcium level, and this may relate to the fact that even minor disruptions in ionic calcium concentrations lead to severe stress and disturbance of calcium balance (Flik et al., 1995).

Unlike terrestrial vertebrates, which depend solely on the diet as calcium source, fish live in an environment which is a readily available source of calcium. Seawater has a calcium concentration of ~10 mmol l^{-1}, whereas the total plasma calcium level of marine fish ranges from 2 to 3 mmol l^{-1}; thus marine fish live in a hypercalcic environment and face an inward gradient of Ca^{2+}. As calcium availability in the environment varies, fish developed calcium regulatory systems which can react rapidly to changes in environmental calcium concentrations (Wendelaar Bonga and Pang, 1991; Björnsson, 1999).

Endocrine control of calcium metabolism in fish is regulated by both hyper- and hypocalcemic hormones. Stanniocalcin (Lafeber et al., 1988; Wagner et al., 1998) acts as the major hypocalcemic (in fact anti-hypercalcemic as it inhibits Ca^{2+} influx) hormone. Increased calcium levels in the medium induce hypercalcemic conditions and by doing so promote stanniocalcin release into the bloodstream, which reduces the calcium influx in the gills and intestine. Prolactin (Kaneko and Hirano, 1993; Mancera et al., 1993; Flik et al., 1994) and PTHrP (parathyroid hormone related protein;

Guerreiro et al., 2001) act as major hypercalcemic hormones. PTHrP is phylogenetically the predecessor of PTH, which appeared only after the water/land transition of vertebrates. Although recent reports indicate that fish express PTH (Danks et al., 2003; Gensure et al., 2004), they also have PTHrP, which has a number of physiological functions, such as bone development, placental calcium transport and cellular growth and development (Martin et al., 1997). In sea bream (*Sparus auratus* L.), PTHrP has been detected in several tissues and plasma by radioimmunoassay using antisera raised against the human peptide (Danks et al., 1993; Devlin et al., 1996) and more recently, against the sea bream peptide (Rotllant et al., 2003). PTHrP has also been found in several other fish species (Ingleton and Danks, 1996; Danks et al., 1998; Trivett et al., 1999, 2001). In addition, hormones such as calcitonin (Wagner, 1997), growth hormone (Flik et al., 1993), vitamin D (Sundell et al., 1992) and cortisol (Flik and Perry, 1989) are known to be involved in the calcium balance of fish.

Sea bream is a euryhaline marine teleost that is important for Mediterranean aquaculture. The intensive culture of this species leads to a high number of morphological malformations that typically result in growth arrest, increased stress sensitivity and an increased incidence of disease outbreaks (Andrades et al., 1996; Carrillo et al., 2001). Improvement of our understanding of calcium regulation is of paramount importance to improve proper development and growth of this species in aquaculture settings.

We investigated calcium regulation after long-term exposure to limited calcium availability. The calcium balance of the fish was monitored through assessment of whole body calcium and phosphorus content, plasma calcium levels and the relationship between calcium and phosphorus accumulation. In this context, we addressed hypercalcemic endocrine factors, *viz* PTHrP and cortisol and investigated their relationship with calcium availability.

The experiments were achieved under controlled laboratory studies where sea bream were exposed to dilute seawater (hypocalcic values of 0.7 mmol l^{-1}) and/or a calcium-deficient diet for prolonged periods of time.

Material and methods

Fish

Juvenile sea bream of approximately one gram mass were obtained from a stock bred at a commercial fish farm (Viveiro Vilanova, Lda., V.N. Milfontes, Portugal). They were transported to the facilities at Radboud University Nijmegen where they were held in an aerated flow through system with 600-litre round tanks at a salinity of 34‰ and a temperature of 23°C. Water quality (pH, NO_2^-, NO_3^-, NH_4^+) was measured once a week and the salinity was checked daily. The photoperiod was 12 h:12 h and the fish stock was fed with commercial pellets daily (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total body mass. The treatment of the fish was in agreement with the Declaration of Helsinki and Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of the Radboud University Nijmegen.

Experimental set-up

To conduct the experiments, the required number of fish was randomly selected from the stock group and transferred to six identical 60-litre round tanks and left to acclimate. After one week, the salinity was lowered from control salinity (34‰; 10.5 mmol l^{-1} calcium) to the test salinity (2.5‰; 0.7 mmol l^{-1} calcium) by continuous flow-through with demineralised water and the diet was gradually changed from the control pellets (Trouvit) to the test pellets (Hope Farms; Woerden, The Netherlands). The calcium-deficient and -sufficient diets were identical in appearance (shape and colour). Although we observed temporary loss of appetite when switching from control to diet pellets, after three days feeding was resumed to comparable levels. This potential problem was addressed by keeping the control diet fish group on a low diet regime (0.5 - 1% food of the total mass) during the adaptation time to the new diet.

In the first experiment, five groups (A-E) of 20 sea bream (start mass: 17.4 ± 4.6 g; N = 20 per group; protandrous fish; not sexually mature) were used. Group A is designated the control group (34‰, control diet); the following test groups were included: group B (34‰, calcium-sufficient diet), group C (34‰, calcium deficient-diet), group D (2.5‰, calcium-sufficient diet) and group E (2.5‰, calcium deficient-diet). The fish were exposed to experimental conditions for six weeks and fasted 24 h before sampling. After

three weeks (t = 1), all fish were weighed and 10 fish were euthanized with 2-phenoxyethanol (1:100; Sigma-Aldrich, St. Louis, MO, USA), freeze-dried until constant mass was reached and subsequently dissolved in concentrated nitric acid (70%; 1 ml g^{-1} dry mass; Sigma-Aldrich) for mineral analyses. Vials were carefully capped to avoid evaporation of the digest and the samples were stored at 4°C. For the second sampling period (after six weeks; t = 2), this procedure was repeated with the remaining fish (N = 10).

For the second experiment, the fish (N = 24 per group) were exposed to experimental conditions for up to nine weeks; sampling took place after three (t = 1), six (t = 2) and nine (t = 3) weeks. At each sampling time, eight fish were randomly selected, euthanized and weighed. Blood was taken from the caudal veins using 1 ml tuberculin syringes, rinsed with Na^+-heparin (Leo Pharma, 5000 U ml^{-1}), five times diluted with demineralised water. Blood thus collected was centrifuged at 13.600 *g* for 10 min. The so obtained plasma was stored at -20°C.

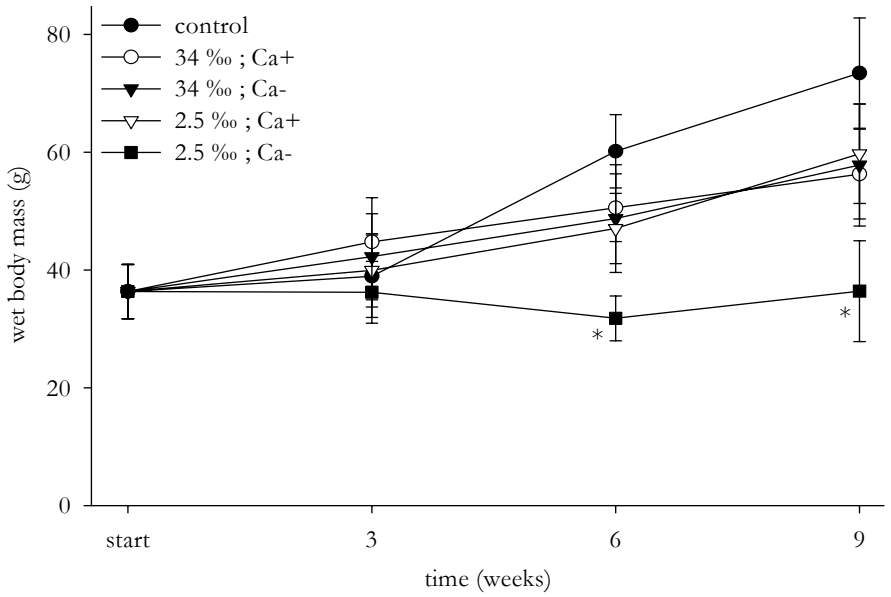

Figure 1. *Growth during nine weeks exposure to calcium-limiting conditions. In the group exposed to limited calcium in both external calcium sources, diet and medium, growth arrest occurs. Values are means ± S.D. Asterisks (*) represent significant difference from control group ($P < 0.05$).*

Whole body mineral concentrations

The nitric acid digests of fish were diluted 1000x with demineralised water and whole body calcium and phosphorus were measured by Inductively Coupled Plasma Atomic Emission Spectrophotometry (ICP-AES, Plasma IL200; Thermo Electron, MA, USA). Mineral concentrations (μmol l^{-1}) of the digests were assessed and content calculated and expressed as μmol g^{-1} dry mass, based on digest total volume and fish dry mass.

In addition to the calcium and phosphorus accumulation rates (μmol h^{-1}), the correlation between the net accumulation of calcium and phosphorus was calculated. Also, the relationship between body mass and whole body calcium (μmol) was determined and the obtained formula of this relationship was used to calculate the whole body calcium levels of the second sampling group at $t = 1$. Data of the measured whole body calcium at $t = 1$ and $t = 2$ and the calculated data of the second group at $t = 1$ were then pooled in full logarithmic plots of the relationship between body mass and whole body calcium at different calcium limiting conditions. This was also done for the relationship between body mass and whole body phosphorus.

Plasma parameters

Plasma Ca^{2+} (mmol l^{-1}) concentration was measured with a Stat Profile pHOx plus analyser (Nova Biomedical, Waltham, USA). Plasma osmolality was measured using a cryoscopic osmometer (Gonotec Osmosat 030, Berlin Germany) and expressed in mOsmol kg^{-1} and plasma total calcium was measured with a calcium kit (Roche, Mannheim, Germany). Plasma cortisol was assessed by radioimmunoassay (RIA) as described by Arends et al. (1999) and plasma PTHrP was measured according to Rotllant et al. (2003).

Statistical analysis

All data were tested for significance by one-way analysis of variance (ANOVA), followed by either Dunn's multiple comparison post hoc test (non-parametric) or the Bonferroni t-test (parametric), where appropriate. Significance was accepted when $P < 0.05$. All values are expressed as mean ± standard deviation (S.D.). Correlation regression between two groups was determined with power function. Because no variation was found in the results of the various parameters between three, six and nine weeks, the data for each parameter were pooled.

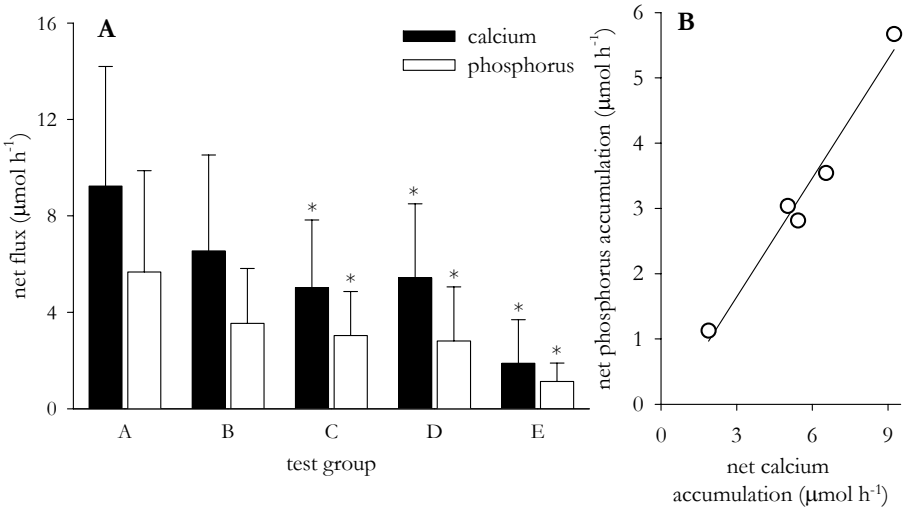

***Figure* 2A.** *Net calcium and phosphorus accumulation (µmol h^{-1}) during three weeks under calcium limited conditions and (**B**) the relationship between net calcium and net phosphorus accumulation (R^2 = 0.94; N = 5; P < 0.01). Values are means ± S.D. Asterisks (*) represent significant difference from control group (P < 0.05).*

Results

Growth

No mortality occurred and all groups ate well during the experiments. Growth of the fish during the nine weeks exposure to experimental conditions is shown in fig. 1. Control fish increased in mass more than the test groups. No growth was observed in group E, which was exposed to both 2.5‰ salinity and a calcium-deficient diet.

Whole body calcium and phosphorus content

Net calcium and phosphorus fluxes in µmol h^{-1} (fig. 2A) and the correlation between calcium and phosphorus accumulation rates (fig. 2B; R^2 = 0.94; N = 5; P < 0.01) demonstrate that net calcium and phosphorus accumulation follow the same pattern, although phosphorus availability was never limited under the experimental conditions. The highest accumulation was observed in the control group (group A); significantly lower net calcium and phosphorus accumulations (groups C and D). Group E exhibited the lowest net calcium and phosphorus accumulation. The fish performed equally well on control and calcium sufficient pellets (groups A and B).

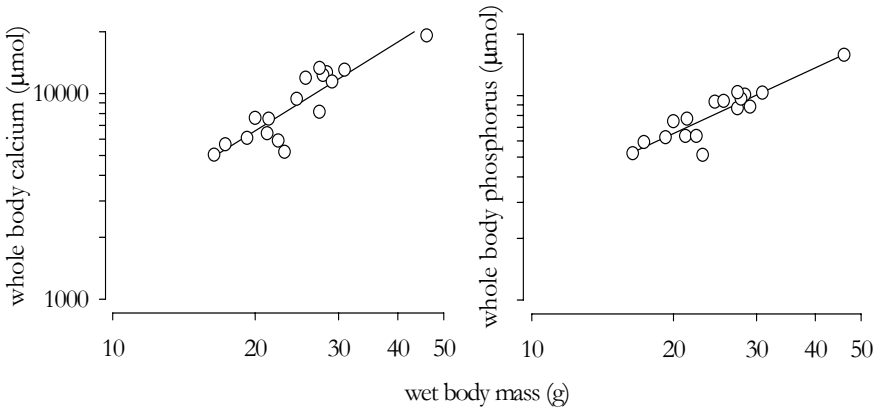

Figure 3. *The relationships between (**A**) wet mass and whole body calcium content (R^2= 0.84, N = 25, p<0.01) and (**B**) wet mass and whole body phosphorus content (R^2 = 0.88, N = 24, P < 0.01) for the control fish. For the test groups, similar correlations were found, although with weaker regression slopes, reflecting a decreased calcium accumulation.*

A logarithmic plot of the relationship between whole body calcium and body mass (M) shows a strong positive correlation (fig 3A; R^2 = 0.84; N = 25; P < 0.01). For the control fish (group A), the relationship is described by the power function $Q = 158.29*M^{1.27}$ (Table 1), where the calculated slope of regression (1.27) reflects the rate of calcium accumulation (Q; µmol) in the fish (Flik et al., 1985; 1993). The test groups show lower power values (plots not shown), with similar regressions in group B and group D. The two groups exposed to calcium-deficient diet (groups C and E) expressed the weakest slopes of regression.

Table 1. *The calculated power functions for relationships between whole body calcium and phosphorus and wet body mass. The regression slopes for calcium and phosphorus decline with limiting calcium availability.*

	Condition		Accumulation power function	
Group	Salinity (‰)	Diet	Calcium	Phosphorus
A	34	control	$Q = 158.29*M^{1.27}$	$Q = 279.81*M^{1.06}$
B	34	calcium sufficient	$Q = 393.17*M^{1.04}$	$Q = 848.67*M^{0.74}$
C	34	calcium deficient	$Q = 645.47*M^{0.85}$	$Q = 576.35*M^{0.83}$
D	2.5	calcium sufficient	$Q = 393.78*M^{1.03}$	$Q = 480.26*M^{0.91}$
E	2.5	calcium deficient	$Q = 560.07*M^{0.91}$	$Q = 570.22*M^{0.83}$

Overall, the power function decreased with lower calcium availability. Similar power functions were made for the relationship between whole body phosphorus and body mass (fig. 3B; $R^2 = 0.88$, $N = 24$; $P < 0.01$). The regression slopes are similar with the slopes that were found for the relationship between calcium and body mass, with the steepest slope in the control group ($Q = 279.81*M^{1.06}$) and weaker phosphorus-body mass regression slopes at calcium limiting conditions (Table 1).

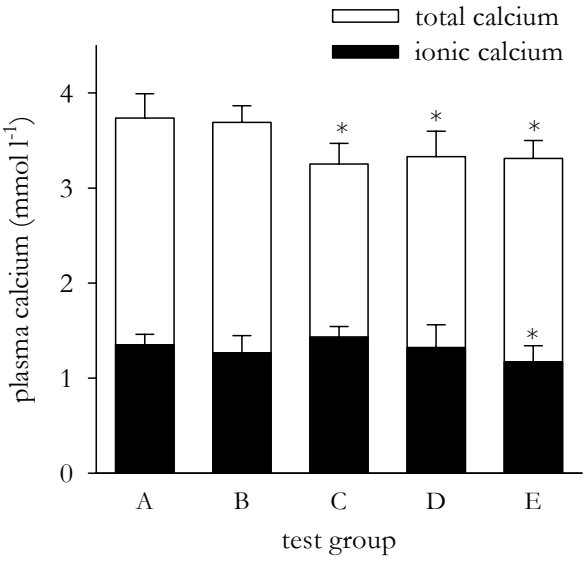

Figure 4. *Total and ionic plasma calcium levels show the more strict control of the ionic calcium concentration compared to the total calcium level. Values are means ± S.D. Asterisks (*) represent significant difference from control group ($p < 0.05$).*

Plasma parameters

In contrast to plasma total calcium levels, which differ significantly in groups where calcium was limited in any way (groups C-E), plasma ionic calcium is strictly regulated, except when fish are fed a calcium-deficient diet and exposed to calcium limited water. Under these conditions, plasma ionic calcium declined significantly.

Plasma cortisol (fig. 5A) is low in controls (6.51 ± 8.78 nmol l^{-1}) and significantly and chronically elevated in the test groups (up to 39.67 ± 12.72 nmol l^{-1}) where calcium access was limited and a decline in total calcium

measured. Plasma PTHrP measurements show concentrations of 0.21 ± 0.06 nmol l^{-1} (fig. 5B) for the control group and higher plasma PTHrP levels of 0.30 ± 0.11 nmol l^{-1} and 0.32 ± 0.12 mmol l^{-1} in groups C and D, exposed to either a calcium deficient-diet or a low salinity, respectively. Group E, exposed to both 2.5‰ and a calcium-deficient diet, expressed a similar PTHrP level as the control group.

For the control group, the positive correlation between plasma PTHrP and plasma ionic calcium is shown in fig. 6. For PTHrP and total calcium no such relationship was found (plot not shown). Also, for the test groups significant correlations were absent.

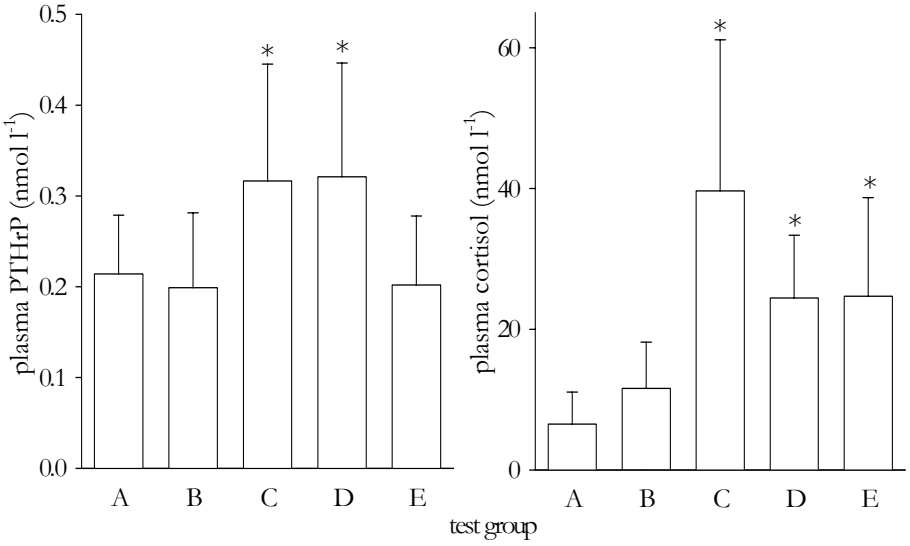

Figure 5A. *Plasma cortisol is shown to increase at calcium limiting conditions when compared with control conditions. (**B**) PTHrP increases when calcium is limited in the diet or the medium. However, when both external calcium sources are limited, PTHrP shows no rise in plasma level. Asterisks (*) represent significant difference (P < 0.05) compared to the control group A.*

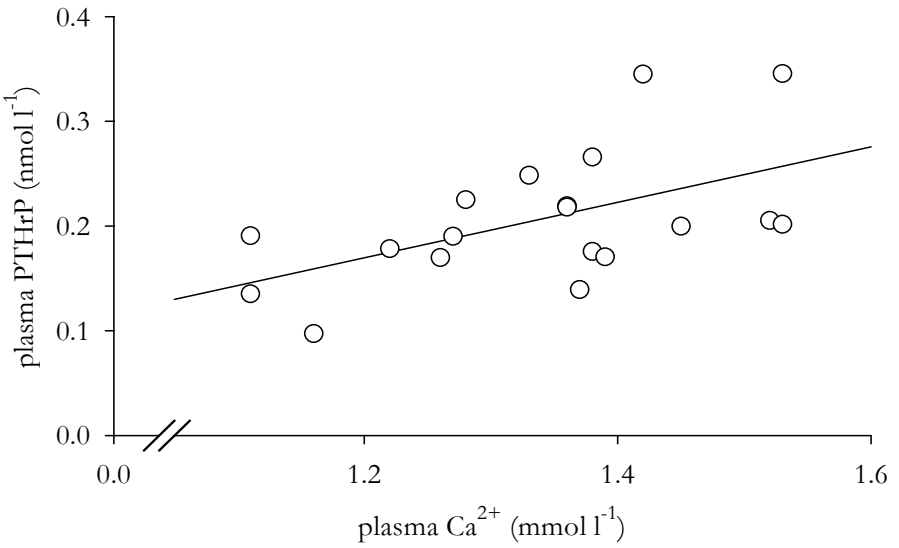

Figure 6. *The positive relationship between plasma PTHrP and ionic calcium ($R^2 = 0.29$; $N = 18$; $P < 0.05$) shows hat PTHrP is involved in the strict control of plasma ionic calcium.*

Discussion

This study provides new observations on prolonged exposure to diluted seawater and/or a calcium deficient diet in sea bream.

1. When growth stops, sea bream still, or with priority, maintain their plasma calcium and in particular the physiologically important free calcium fraction at a concentration that ensures their survival for a prolonged period of time. Strong relationships were found between body mass and whole body calcium and phosphorus for all groups tested, with decreasing slopes (decreasing whole body calcium and phosphorus content) under decreasing calcium availability in water and diet.

2. Net calcium and phosphorus accumulation rates decline when calcium is limited. A strong positive correlation was found between net calcium and phosphorus accumulation, although phosphorus was not limited in the experimental set-up.

3. In control fish, a positive correlation was found between plasma PTHrP and ionic calcium concentrations.

4. Plasma ionic calcium levels are strictly regulated, whereas total plasma calcium levels show significant differences under calcium-limiting conditions. Interestingly, when hypocalcemia was observed, plasma cortisol and PTHrP levels were mildly increased, which we take as an indication for a hypercalcemic action or function of these hormones. The mild endocrine responses concur with an allostasis concept, where these mild elevations would represent a normal allostatic load (McEwen and Wingfield, 2003).

Whole body calcium

With respect to the calcium balance, prolonged exposure to diluted seawater (2.5‰, which is a hypocalcemic medium) and a calcium-deficient diet results in growth arrest in sea bream. This phenomenon has been described for several other teleost species (Flik, 1986; Morgan and Iwama, 1991; Woo and Kelly, 1995; Sampaio and Bianchini, 2002). Interestingly, the apparent growth arrest allows the fish to maintain plasma calcium balanced at a level that ensures their survival for prolonged times. Apparently, the calcium stores realised under control conditions, have a significant buffer capacity. We calculate for 50 gram sea bream total calcium content of 29.2 mmol under control conditions and of 16 mmol when water and diet are low in calcium. This indicates a 42% decrease in the total calcium pool. Such drastically lower calcium content may be possible only in aquatic vertebrates.

Plasma calcium

Ionic calcium levels are strictly regulated and fish are able to maintain this physiologically important free calcium level when calcium availability is reduced in the diet and/or the medium. However, when calcium availability is strongly reduced in both of the external calcium sources (the diet and the medium), a slight, but significant decrease in ionic calcium is observed. The strict control of ionic calcium means that the calcemic regulation system must be able to react swiftly on variable external calcium availability. A positive correlation between the hypercalcemic hormone PTHrP and ionic calcium indeed is found. This indicates that PTHrP is involved in the calcemic endocrine control of plasma calcium balance in fish. Total calcium is not as tightly regulated as ionic calcium by the calcemic control mechanisms, which means larger variations in plasma total calcium

concentration are found, indicating a change in binding protein level compared with the control group. Indeed, no positive relation between plasma total calcium and plasma PTHrP is found here.

Calcium and phosphorus accumulation

The positive correlation found between body mass and whole body calcium is not affected by severe and chronic decreases in external calcium availability. A similar relationship was found between body mass and whole body phosphorus for all experimental conditions. This is remarkable, because the experimental conditions were focussed on calcium-limiting conditions, with phosphorus concentrations unaffected. Since the phosphorus concentration in sea water is very low, fish must depend on their diet for phosphorus which they accumulate at the rate of that for calcium (Roy and Lall, 2003). Yet, we have demonstrated that phosphorus accumulation is impeded under conditions of low calcium availability (Vielma and Lall, 1998; Chavez-Sanchez et al., 2000). Indeed, intestinal adsorption of phosphorus has been shown to be coupled to calcium adsorption in a variety of vertebrates (Mol et al., 1999). These studies mainly focus on the relationship between calcium and phosphorus in relation to availability in diet and or medium and subsequently growth. In the present study, we observed growth arrest under limited calcium concentrations. Since most of the whole body calcium and phosphorus is incorporated in bone and scales as calcium phosphate and calcium carbonate complexes, growth arrest due to calcium-limiting conditions apparently also leads to a subsequent decrease in net phosphorus influx.

PTHrP and cortisol

So far, only limited information is available on plasma PTHrP in sea bream. Danks et al. (1993) measured PTHrP in sea bream plasma and found 12.43 ± 1.48 pmol l^{-1}. Here, we present PTHrP values of 0.21 ± 0.06 to 0.32 ± 0.12 nmol l^{-1}. These values are in line with the values reported by Rotllant et al. (2003), where, using the same RIA as in this study, PTHrP values of 2.5 ± 0.29 ng ml^{-1} (0.61 ± 0.07 nmol l^{-1}) in 100-150 g fish were found. The lower values reported may well be caused by the lower immunoreactivity of the heterologous antisera with fish PTHrP, explained by different amino acids in

the human N-terminal PTHrP sequence compared with fish consensus (discussed by Rotllant et al., 2003).

The plasma PTHrP levels in the two groups that were exposed to either a calcium-deficient diet or a diluted medium show a significant increase when compared with the plasma PTHrP level of the control group. However, when calcium was limited in both diet and medium, plasma PTHrP level did not increase compared to the control fish. A possible explanation for this is that the results show that growth, although decreased, is continuing in the groups in which the fish still had access to a natural calcium source, either in the diet or medium. For this growth, a positive net calcium accumulation is required, (which may well be supported by a hypercalcemic action of PTHrP) which is confirmed by our results. On the other hand, the fish in group E show growth arrest during the experiment. The net calcium flux in this group was a 4.5-fold lower compared to the control group and 2- to 3-fold compared to the other test groups. Under their apparent growth arrest, no net calcium influx for skeletal formation is required. Apparently, the calcemic endocrine system successfully controls blood plasma calcium levels to a level that ensures proper physiology and survival of the fish.

Cortisol values are approximately two times higher in the 2.5‰ group and 3-4 times higher in the calcium-deficient diet groups than in the control group. Although significantly higher, these values still do not exceed the basal level documented for this species, indicating that the fish were not stressed. Arends et al. (1999) measured basal cortisol levels of 25 nmol l^{-1} in sea bream. These values are in the same range as the basal levels in our experiment. It has been shown before that subtle differences in basal cortisol levels could account for changes in osmolality, Na^+/K^+-ATPase and plasma calcium levels (Metz et al., 2003). Flik and Perry (1989) demonstrated increased cortisol secretion during hypocalcemic stress in freshwater rainbow trout, inducing the uptake of calcium ions from the water by regulating the Ca^{2+} pumps in the gills. Also, elevated plasma cortisol levels have been shown to play a role in hypo-osmotic adaptation. Mancera et al. (1994) showed increased cortisol levels in sea bream after transfer from 39‰ to brackish water of 7‰. The results reported here are corroborated by these early findings.

In the present study, we have demonstrated that sea bream can cope well with limited calcium availability in either diet or medium. The fish continued to grow and up-regulated hypercalcemic hormones PTHrP and cortisol, allow the fish to maintain the physiologically important ionic calcium level constant.

In case of limiting calcium availability in both external calcium sources, growth arrest occurs in sea bream and whole body calcium level can be so maintained that no large net calcium accumulation is needed for skeletal formation. The relatively small net calcium accumulation rate that is still achieved by the fish can thus be used to maintain plasma calcium balance in such a way that it ensures the survival of the fish for a prolonged period of time.

Acknowledgements

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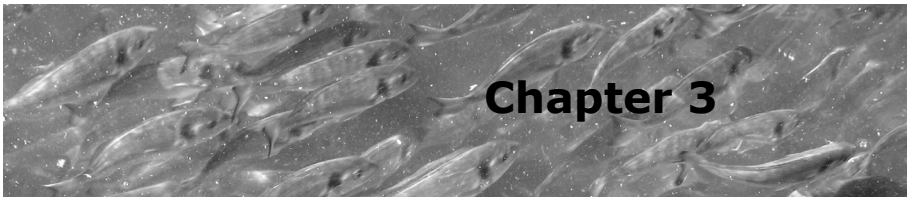

Chapter 3

PTHrP regulation and calcium balance in sea bream (*Sparus auratus* L.) under calcium constraint

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Abstract

Juvenile gilthead sea bream were exposed to diluted seawater (2.5‰ salinity; DSW) for 3 h or, in a second experiment, acclimated to DSW and fed a control or calcium-deficient diet for 30 days. Branchial Ca^{2+} influx, drinking rate and plasma calcium levels were assessed. Sea bream Parathyroid Hormone-related Protein (sPTHrP) was measured in plasma and mRNAs of pthrp, its main receptor, pth1r and the calcium-sensing receptor (casr) were quantified in osmoregulatory tissues and the pituitary gland. When calcium is limited in water or diet, sea bream maintain calcium balance; however, both plasma Ca^{2+} and plasma sPTHrP concentrations were lower when calcium was restricted in both water and diet. Positive correlations between plasma sPTHrP and plasma Ca^{2+} ($R^2 = 0.30$; $N = 39$; $P < 0.05$) and plasma sPTHrP and body mass of the fish ($R^2 = 0.37$, $N = 148$, $P < 0.001$) were found. Immunoreactive sPTHrP was demonstrated in pituitary gland pars intermedia cells that border the pars nervosa and co-localises with somatolactin. In the pituitary gland, pthrp, pth1r and casr mRNAs were down-regulated after both short- and long-term exposure to DSW. A correlation between pituitary gland pthrp mRNA expression and plasma Ca^{2+} ($R^2 = 0.71$; $N = 7$; $P < 0.01$) was observed. In gill tissue, pthrp and pth1r mRNAs were significantly up-regulated after 30 days exposure to DSW, whereas no effect was found for casr mRNA expression. We conclude that in water of low salinity, declining pituitary gland pthrp mRNA expression accompanied by constant plasma sPTHrP levels points to a reduced sPTHrP turnover and that sPTHrP, through paracrine interaction, is involved in the regulation of branchial calcium handling, independently of endocrine pituitary gland sPTHrP.

Introduction

Parathyroid Hormone-related Protein (PTHrP) is a hypercalcemic factor in fish (Guerreiro et al., 2001). Phylogenetically it is the ancestor of parathyroid hormone (PTH), which is the major hypercalcemic hormone in terrestrial vertebrates. A sea bream (*Sparus auratus* L.) *pthrp* cDNA has been cloned (Flanagan et al., 2000) and the genomic structure of *pthrp* was clarified in fugu (*Fugu rubripes*, Temminck and Schlegel, 1850; Power et al., 2000). Recently, *pth* genes were discovered in the zebrafish (*Danio rerio*, Hamilton, 1822) genome (Gensure et al., 2004). The two hormones share a high N-terminal amino acid sequence homology and both peptides bind and activate shared G-coupled PTH/PTHrP receptors (Gardella and Jüppner, 2001). Three different PTHrP receptors were identified in fish (PTH1R, PTH2R and PTH3R), of which PTH1R is the most common and shares homologous parts with the mammalian PTH1R (Rubin and Jüppner, 1999). PTHrP has a key function in several physiological and biochemical processes in fish, including tissue differentiation and proliferation, vitellogenesis (Guerreiro et al., 2002; Bevelander et al., 2006), cortisol production (Rotllant et al., 2005a), calcium regulation (Guerreiro et al., 2001; Abbink et al., 2004) and calcium resorption from bone and scales (Rotllant et al., 2005b), which strongly indicates that PTHrP is involved in (skeletal) calcium physiology. The presence of PTHrP in a large number of tissues suggests PTHrP to be an auto-/intra- or paracrine factor. However, the immunohistochemical detection of PTHrP in the pituitary gland could also suggest a classical endocrine function for PTHrP in fish, as suggested by Danks et al. (1993).

Fish have access to infinite sources of readily available calcium in the water. Calcium present in water and diet can be taken up *via* gills and intestine and calcium balance is achieved by branchial efflux and intestinal excretion. About 99% of the total calcium in fish is incorporated in skeleton and dermal scales (Flik et al., 1986). The latter have a protective function, but also serve as an internal calcium buffer. In fish blood, the plasma total calcium concentration is about 2–3 mmol l^{-1} of which the ionic fraction accounts for about half (Hanssen et al., 1991). This ionic fraction is important for numerous physiological and biochemical processes and is therefore tightly regulated within narrow limits by calcemic endocrines (Flik et al., 1995). As the calcium availability in water and diet vary, as does the need for

calcium, the calcemic endocrine system should react swiftly to changes in calcium availability or need (Björnsson et al., 1999).

This study focused on the regulation of the hypercalcemic sPTHrP and the calcium balance in response to a short- and long-term calcium constraint in water and/or diet. Juvenile sea bream were rapidly transferred from full strength seawater (SW; 34‰ salinity; 10.5 mmol l^{-1} Ca^{2+}) to diluted sea water (DSW; 2.5‰ salinity; 0.7 mmol l^{-1} Ca^{2+}) and sampled three hours later (the *short-term experiment*). In a second experiment, juvenile sea bream were exposed to SW or DSW and were fed a calcium sufficient (Ca+ diet) or calcium deficient diet (Ca- diet) for 30 days (the *long-term experiment*). The experiments were achieved under controlled laboratory conditions. Gill Ca^{2+} influx ($F_{in}Ca^{2+}$), drinking rate (*DR)*, plasma sPTHrP, as well as plasma total and ionic calcium concentrations were assessed. Expression for *pthrp, pth1r* and *casr* mRNA were quantified in gill, intestine, kidney and the pituitary gland and immunostaining was used to examine sea bream pituitary glands for sPTHrP immunoreactivity.

Material and methods

Fish

Juvenile gilthead sea bream (*Sparus auratus* L.) were obtained from a commercial fish farm (Viveiro Vilanova, Lda., V. N. Milfontes, Portugal) and flown to The Netherlands without mortality. The fish were kept in round 600-litre tanks with aerated flow through, a salinity of 34‰ and a temperature of 23 ± 1°C. The fish were fed commercial pellets (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total body mass daily. The treatment of the fish was in agreement with the Declaration of Helsinki and Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of the Radboud University Nijmegen.

Experimental set-up

Short-term experiment: Fourteen fish were placed in a tank with identical water conditions as in the stock. After one week of acclimatisation, the fish were rapidly transferred to a second tank with identical conditions (SW; control transfer; N = 7) or to a tank containing diluted seawater of 2.5‰ salinity (DSW; N = 7). After 3 h, the fish were euthanized with 2-phenoxyethanol (1:200; Sigma-Aldrich, St Louis, MO, USA) and blood was

taken from the caudal vessels using a 1 ml tuberculin syringe, rinsed with 5x diluted Na^+ heparin (Leo Pharma, Weesp, The Netherlands, 1000 U ml^{-1}). The collected blood was centrifuged at 13.600 *g* for 10 minutes and the plasma so obtained stored at –20°C. Fish were not fed for 24 h before sampling.

Long-term experiment: Fish (N = 160) were randomly selected from stock, placed in four round tanks with 40 fish per tank and left to acclimate. After one week, the salinity was gradually lowered by continuous flow-through with demineralised water until test salinity of 2.5‰ (0.7 mmol l^{-1} Ca^{2+}) was reached, after 48 hours. The diet was changed from control pellets to the test pellets (Hope Farms, Woerden, The Netherlands). The experimental animals were fed first and the controls received an equivalent amount of food as taken up by the experimentals. After three days, the fish fully accepted the new diet and ate all the food provided (2% of the total body mass daily). The four groups of fish included a control (group a: 34‰ salinity; Ca+ diet) and three test groups: Group b (34‰ salinity; Ca– diet), group c (2.5‰ salinity; Ca+ diet) and group d (2.5‰ salinity; Ca– diet). After 30 days, the fish were sampled as described for the short-term experiment; on the day before sampling, feeding was discontinued.

Calcium influx and drinking

After 30 days into the experiment, 20 fish from each group were randomly selected and placed in two identical vessels. After 24 h of acclimation, ^{51}Cr-EDTA (1.9 kBq ml^{-1}) or $^{45}CaCl_2$ (2.5 kBq ml^{-1}) was added to the tanks to assess drinking rate (*DR*) and gill Ca^{2+} influx ($F_{in}Ca^{2+}$), respectively (Flik et al., 1985). Fish were sampled 2 h (*DR*) or 4 h ($F_{in}Ca^{2+}$) after addition of the isotopes. Water samples were collected and the fish were euthanized by adding 2-phenoxyethanol (1:200; Sigma-Aldrich) to the water. The fish were rinsed with demineralised water, quick-frozen in solid CO_2 and the frozen intestinal track was removed. Samples were weighed and rapidly digested in H_2O_2 (35%; 2 ml g^{-1}; Lamers & Pleuger, 's Hertogenbosch, The Netherlands). Water calcium content was measured with a calcium kit (Roche, Mannheim, Germany; cat. nr. 1489216) and radioactivity in the water and digested fish samples was counted with a liquid scintillation counter (Wallac 1410; Wallac, Turku, Finland). An OptiPhase HiSafe 3 liquid

scintillation cocktail (Perkin-Elmer, Boston, MA, USA) was added before counting.

DR was calculated as: $DR = A_i / (A_w\, t\, m)$, where A_i is the total activity of ^{51}Cr-EDTA in the intestinal track (c.p.m), A_w is the total activity in the water (c.p.m. nl^{-1}), t is the exposure time to ^{51}Cr-EDTA (h) and m is the mass (mg) of the fish (Flik et al., 2002).

$F_{in}^{Ca^{2+}}$ was calculated as: $F_{in}^{Ca^{2+}} = (A_f\, C_w) / (A_w\, t)$, where A_f is the total activity of $^{45}Ca^{2+}$ in the fish (d.p.m.), C_w is the calcium concentration in the water (pmol l^{-1}), A_w is the total activity in the water (d.p.m. l^{-1}), and t is the duration of exposure to $^{45}Ca^{2+}$ (h). Since there were no differences in total mass between groups, the results are expressed as nl h^{-1} (*DR*) and nmol h^{-1} ($F_{in}^{Ca^{2+}}$) and were not normalized for body mass (Guerreiro et al., 2004).

Plasma analyses

Plasma Ca^{2+} (mmol l^{-1}) was measured using a Stat Profile pHOx plus analyser (Nova Biomedical, Waltham, MA, USA). Plasma total calcium (mmol l^{-1}) was assessed using a calcium kit (Roche, Mannheim, Germany) and plasma PTHrP (nmol l^{-1}) was measured with a homologous radioimmunoassay according to Rotllant et al. (2003).

Immunohistochemistry

Juvenile sea bream pituitary glands were fixed in Bouin's fixative for 90 min, dehydrated and embedded in paraffin wax. Sections were cut at 5 μm and dewaxed using xylene and degraded alcohols. The immunostaining procedure followed the protocols earlier described for PTHrP (Danks et al., 1993) and somatolactin (SL; Kaneko et al., 1993). Rabbit anti-sea bream (1-34)sPTHrP (1:100) and rabbit anti-rainbow trout SL (1:3000; a gift of Dr. Sho Kakizawa, Ocean Research Institute, Tokyo) were used as primary antibodies. For sPTHrP immunostaining, the sensitive immunoperoxidase method with the Vectastain avidin–biotinylated enzyme complex (Vectastain ABC; Vector Laboratories Inc., Burlingame, CA, USA) was used to increase the staining intensity. Periodic-acid Schiff (PAS) staining was used to distinguish the two cell populations of the pars intermedia (pi) of the pituitary gland, the PAS-positive somatolactin cells and the PAS-negative melanocyte-stimulating hormone (MSH) cells.

Expression of pthrp, pth1r and casr mRNA

Tissue samples from the gill, kidney, anterior part of the intestine and the pituitary gland were collected. Quantitative real-time, PCR (RT-PCR) was used to quantify the mRNA expression levels for *pthrp*, *pth1r* and the *casr* according to Hang et al. (2005), with the housekeeping gene β-actin as endogenous control.

Statistics

All data are expressed as mean ± standard deviation (S.D.); differences among groups were assessed by ANOVA. Significance of differences was assessed by parametric (Student's t-test) or non-parametric (Mann–Whitney *U*-test) when appropriate and $P<0.05$ was taken as fiducial limit.

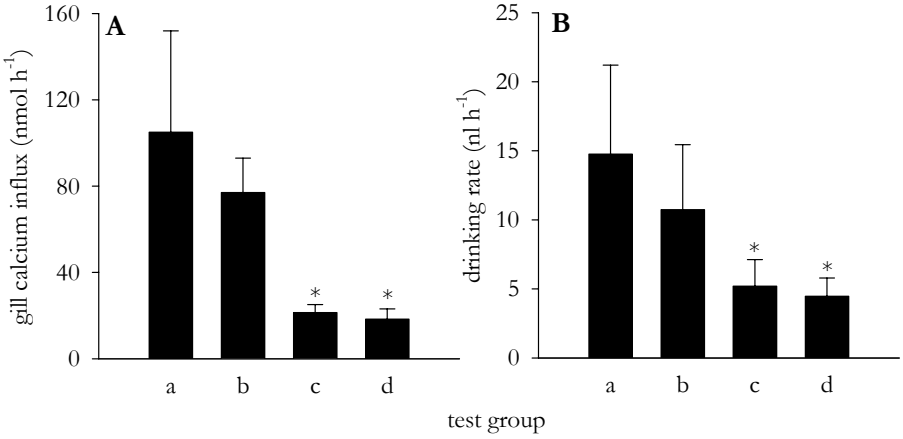

Figure 1. *Gill $F_{in}^{Ca^{2+}}$ (N = 10; **Fig. 1A**) and DR (N = 10; **Fig. 1B**) had significantly decreased in fish exposed to DSW for 30 days. The four groups of fish are a control (group a: 34‰ salinity; Ca+ diet) and three test groups: Group b (34‰ salinity; Ca- diet), group c (2.5‰ salinity; Ca+ diet) and group d (2.5‰ salinity; Ca- diet). Asterisks indicate significant difference (P < 0.05) when compared with control values. Values are means ± S.D.*

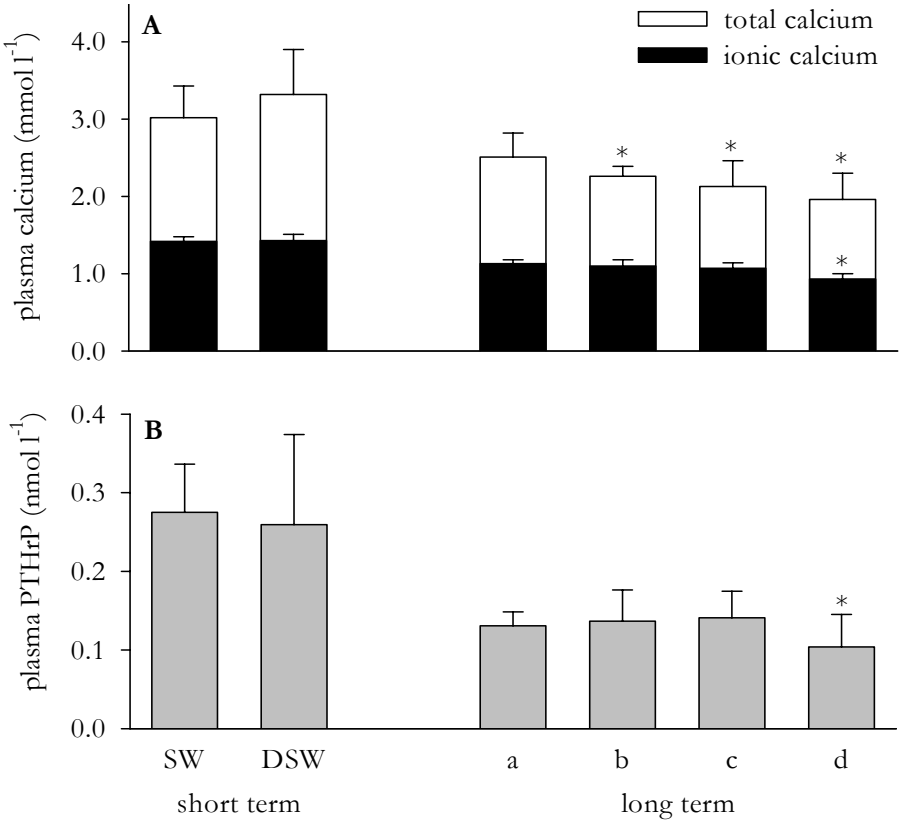

Figure 2A. *Plasma calcium levels (N = 7 for the short-term experiment and N = 10 for the long-term experiment) show the strict control of the ionic fraction, which had only slightly, but significantly decreased after long-term calcium constraint in both water and diet. In accordance, plasma sPTHrP decreased in group d compared to controls (**2B**). Asterisks indicate significant difference (P < 0.05) from control group a. Values are means ± S.D.*

Results

Gill calcium influx and drinking rate

Fig. 1A shows that branchial $F_{in}^{Ca^{2+}}$ declined significantly from 105 ± 47 and 77 ± 17 nmol h^{-1} in the SW groups a and b to 21.4 ± 3.7 and 18.0 ± 4.8 nmol h^{-1} in the DSW groups c and d respectively. This same pattern was found for drinking (Fig. 1B), with a decrease from 14.8 ± 6.5 and 10.7 ± 4.7 nl h^{-1} in SW to 5.2 ± 1.93 and 4.5 ± 1.3 nl h^{-1} in the DSW groups. Modification of calcium in the diet had no effect on gill $F_{in}^{Ca^{2+}}$ or *DR*.

Plasma analyses

Exposure to DSW for 3 h had no effect on the total and ionic plasma calcium level (Fig. 2A). When calcium was limited in water and/or diet for 30 days, the plasma total calcium concentration decreased in all experimental groups, whereas the ionic fraction decreased only when calcium was limited in both water and diet (1.13 ± 0.05 mmol l^{-1} Ca^{2+} in group a and 0.93 ± 0.07 mmol·l^{-1} Ca^{2+} in group d).

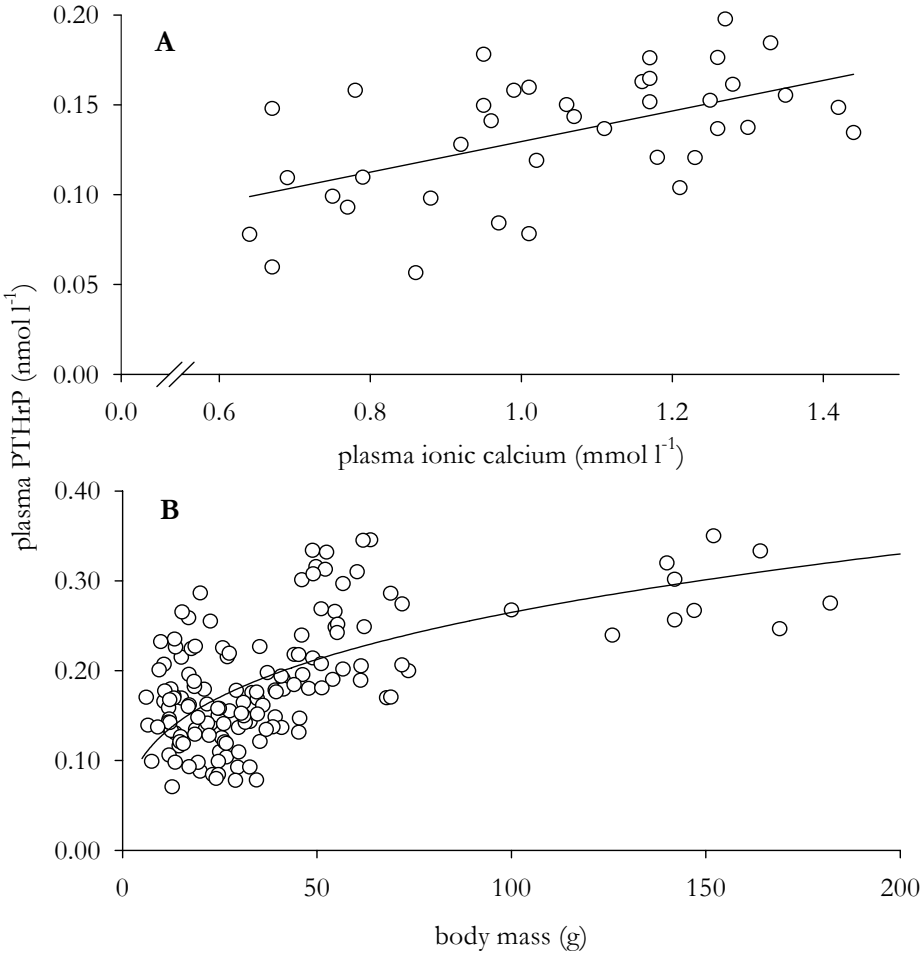

***Figure 3**. Positive relationships were found between plasma sPTHrP and plasma ionic calcium (linear regression; **Fig. 3A**; $R^2 = 0.30$; $N = 39$; $P < 0.05$) and between plasma sPTHrP and the body weight of the fish (power function; **Fig. 3B**; $R^2 = 0.37$, $N = 148$, $P < 0.001$).*

The plasma sPTHrP level was not affected after exposure to DSW for 3 h (Fig. 2b), measuring 0.28 ± 0.06 nmol l^{-1} in the controls (group a) and 0.26 ± 0.12 nmol l^{-1} sPTHrP in the DSW fish. In the long-term experiment, the plasma sPTHrP level had slightly, but significantly, decreased when calcium was limited in both water and diet, from 0.13 ± 0.02 nmol l^{-1} in the controls to 0.10 ± 0.04 nmol l^{-1} in group d (Fig. 2B). The fish from the short-term experiment were transferred to another tank 3 h before sampling, which could have induced a stress response and thus increased cortisol levels. The measured cortisol levels, 42.1 ± 10.1 nmol l^{-1} (N = 7) in the SW fish and 38.5 ± 10.8 nmol l^{-1} (N = 7) in the DSW fish, do not represent stress levels in sea bream (Arends et al., 1999). We thus exclude stress-induced elevated cortisol levels as a cause of the difference seen in sPTHrP levels between the short- and long-term experiments. These differences may relate to different body weights of the groups in the short and long term experiment.

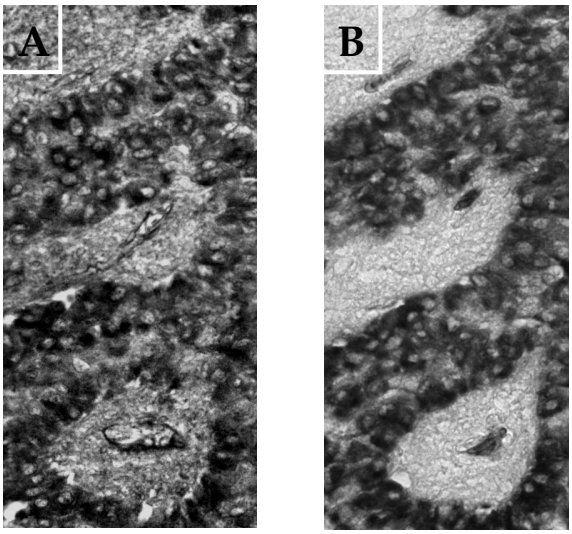

Figure 4. *Immunostaining for (1-34)sPTHrP (**Fig. 4A**) and SL (**Fig. 4B**) in sea bream pituitary glands shows the co-localisation for sPTHrP and SL in cells of the pars intermedia, bordering the pars nervosa; magnification: 400x. Controls with omission of the first antibody and pre-absorption with sPTHrP confirmed the specificity of sPTHrP immunoreactivity.*

Fig. 3 shows the correlation that was found between plasma Ca^{2+} and plasma sPTHrP (Fig. 3A; $R^2 = 0.30$; $N = 39$; $P < 0.05$) and between plasma sPTHrP and the wet weight of the fish (Fig. 3B; $R^2 = 0.37$, $N = 148$, $P < 0.001$) for all the control observations made.

Immunohistochemistry

Immunostaining with antisera to sea bream (1-34)sPTHrP and trout (*Oncorhynchus mykiss* Walbaum, 1792) SL revealed immunoreactive sPTHrP and SL in pituitary gland pi cells (Fig. 4). The sPTHrP-immunoreactivity was found in the PAS-positive SL cells and bordered the pars nervosa (pn). Controls with omission of the first antiserum and pre-absorption with sPTHrP confirmed the specificity of sPTHrP immunoreactivity (data not shown).

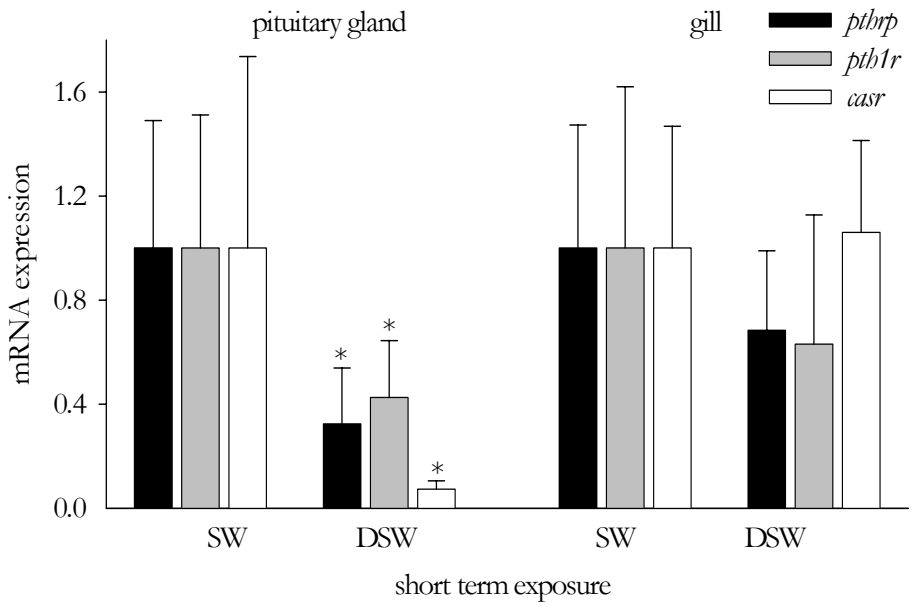

Figure 5. *Expression for pthrp, pth1r and casr mRNA (N = 7) in the pituitary gland is down-regulated after three hours of exposure to DSW, whereas no effect was found in gill tissue. Asterisks indicate significant difference (P < 0.05) compared to SW values.*

Pthrp, pth1r and casr mRNA expression

Expression of *pthrp, pth1r* and *casr* mRNA was significantly down-regulated in the pituitary gland of fish exposed to DSW for 3 h (Fig. 5). In kidney, intestine and gill, no effect on *pthrp, pth1r* or *casr* mRNA expression was observed (data for kidney and intestine not shown).

Exposure to DSW for 30 days resulted in significant down-regulation of *pthrp, pth1r* and *casr* mRNAs in the pituitary gland when compared with the control group (Fig. 6A). In gills, a significant up-regulation of *pthrp* and *pth1r* mRNA was found in the DSW fish, whereas mRNA expression for *casr* remained constant (Fig. 6B). In intestine and kidney, no differences in expression of *pthrp, pth1r* and *casr* mRNA were seen (data not shown).

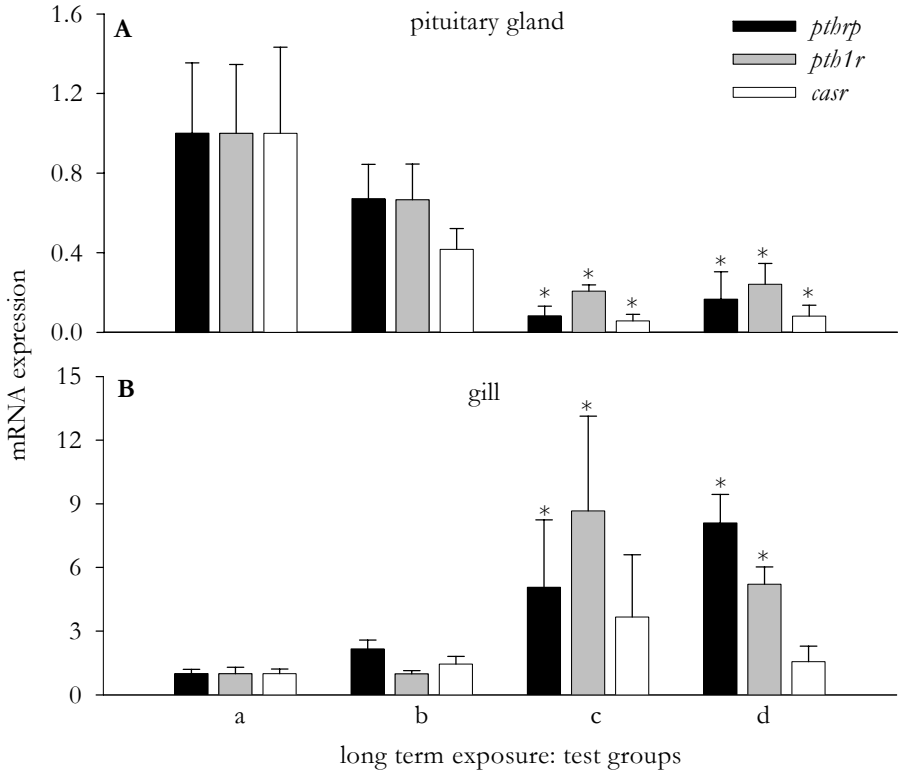

Figure 6. *Expression for pthrp, pth1r and casr mRNA (N = 7) in the pituitary gland (**A**) is down-regulated after three weeks of exposure to DSW, whereas in gills, an up-regulation was observed (**B**). Asterisks indicate significant difference (P < 0.05) compared to SW values.*

In the short-term experiment, a strong relationship between plasma Ca^{2+} and pituitary gland *pthrp* mRNA expression was observed in the controls (Fig. 7; $R^2 = 0.71$; $N = 7$; $P < 0.01$), whereas in the DSW group, this relationship was absent. No such samples were available in the long-term experiment.

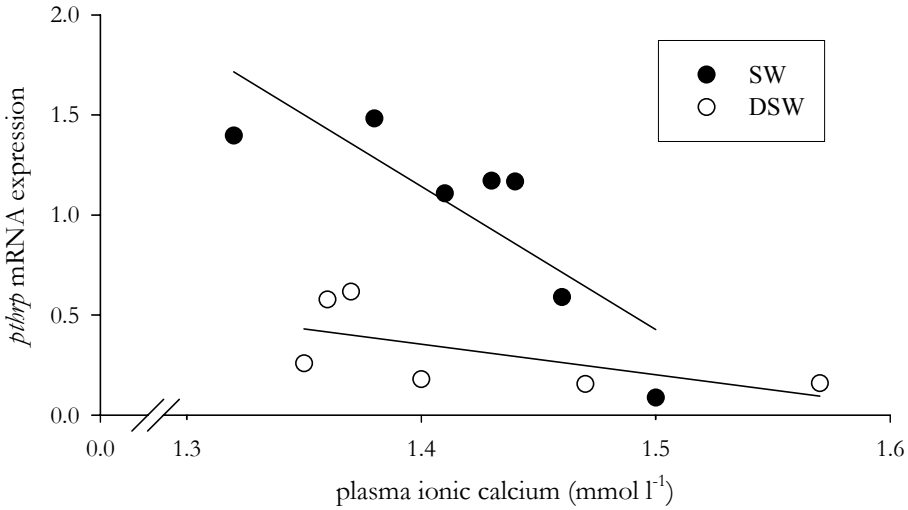

Figure 7. *In the short-term experiment, a negative correlation between pituitary gland pthrp expression and plasma Ca^{2+} was found in the SW fish ($R^2 = 0.71$; $N = 7$; $P < 0.01$). In the DSW fish, no relation was found. For the long-term experiment, no such data were available.*

Discussion

This study provides new findings about the regulatory role of sPTHrP in the calcium balance of juvenile sea bream under calcium constraint:

-Branchial $F_{in}^{Ca^{2+}}$ and *DR* decreased in DSW; plasma Ca^{2+} was slightly, but significantly, decreased after long-term exposure to limited calcium in both water and diet. Apparently, DSW induces mild hypocalcemia, which is not counteracted by increased Ca^{2+} uptake from the water or by drinking.

- Plasma sPTHrP level decreased after long-term limitation of calcium in both water and diet and plasma sPTHrP correlated with plasma Ca^{2+} and the

body mass of the fish. This points to a decreased calcium turnover under calcium constraint.
- Immunoreactive sPTHrP co-localised with immunoreactive SL in PAS-positive cells of the pi in the pituitary gland, indicating that pituitary sPTHrP is the source of the high plasma sPTHrP levels in the fish.
- In the pituitary gland, down-regulation of *pthrp, pth1r* and *casr* mRNA was observed after both 3 h and 30 days of calcium constraint. In gills, mRNA for *pthrp* and *pth1r* was up-regulated, but only after 30 days, whereas *casr* mRNA expression was not affected by calcium restraint. Thus, we have evidence for a branchial sPTHrP regulatory system, acting independently of endocrine pituitary gland sPTHrP actions. The branchial chloride cell, being a main factor in calcium uptake in fish (Flik et al., 1995), appears to be a component of a para-/auto- or intracrine hypercalcemic hormonal control mechanism. The production of sPTHrP by the chloride cell may be adjusted *via* CaSR activity. *In situ* hybridisation experiments are required for confirmation.

Gill $F_{in}^{Ca^{2+}}$ and DR

For fish, seawater is a strongly hypercalcic environment (~10 mmol l^{-1} Ca^{2+}) and therefore, fish are forced to reduce Ca^{2+} influx or to actively secrete Ca^{2+} to compensate for excessive Ca^{2+} influx. To compensate for osmotic water loss, sea water fish constantly drink, which at the same time represents a high Ca^{2+} load. The transepithelial potential in fish is always more positive than the equilibrium potential for Ca^{2+} across the integument and therefore, the electrochemical gradient for Ca^{2+} (the driving force for passive Ca^{2+} movement across the gills), is directed outwards (+30 mV), causing a substantial passive Ca^{2+} efflux over the leaky branchial epithelium (Flik and Verbost, 1993). The uptake of Ca^{2+} is therefore not by diffusion, but by active transport.

The DSW (0.7 mmol l^{-1} Ca^{2+}) causes a decrease in osmotic exchange, which subsequently results in the measured three fold decline in *DR* (and a consequent decrease in Ca^{2+} intake through drinking) and a five-fold decline in gill $F_{in}^{Ca^{2+}}$. In a hypotonic solution such as DSW, the osmotic water loss reverses to water gain and the influx of Ca^{2+} occurs almost entirely *via* the gills (Flik et al., 1986). The 15 times lower ambient Ca^{2+} concentration

combined with a just five fold decline in $F_{in}Ca^{2+}$ points to an increase in Ca^{2+} influx capacity or efficiency. This could be achieved by an increase in chloride cell density in the branchial epithelium (Flik et al., 1986) and enhanced prolactin (PRL) secretion. Low salinity is known to increase PRL secretion from the pituitary gland (Kaneko and Hirano, 1993) in salt water fish. PRL is known to limit ionic losses and water permeability in osmoregulatory tissues in hypo-osmotic media and to stimulate Ca^{2+} influx through gills and Ca^{2+}-ATPase activity in gill plasma membranes (Flik et al., 1994), thereby increasing the Ca^{2+} influx capacity. The hypercalcemic control by sPTHrP which is shown in this study may connect both factors. This idea is further strengthened by the observation, that gene expression for *pthrp* in mammals is up-regulated in response to increased plasma PRL levels (Thiede, 1989).

Endocrinology

An interesting observation is the relationship between the wet body mass of the fish and the plasma sPTHrP concentration. In juvenile sea bream, the plasma sPTHrP level increases with the body weight and plateaus with increasing weight of the fish, which suggests a decreasing need for hypercalcemic control with increasing body mass. Apparently, hypercalcemic control in juvenile stages is critically dependent on sPTHrP. As the growth rate of fish decreases with age, the need for calcium to be incorporated into the skeleton and scales also decreases, as does the requirement for regulatory sPTHrP. Strong positive correlations between plasma sPTHrP and the whole body content of calcium, phosphorus and magnesium (the main minerals in bone) were found (Abbink et al., 2007), which strengthens the assumption that sPTHrP is involved in skeletal calcium physiology. This assumption was recently forwarded by Redruello et al. (2005), who showed a down-regulation of the unique matricellular calcium binding glycoprotein osteonectin by PTHrP and by Rotllant et al. (2005b), who reported that PTHrP induced osteoclastic activity in scale tissue, indicated by its stimulation of tartrate-resistant acid phosphatase (TRAPC, a marker for osteoclastic activity).

Immunohistochemistry

In the sea bream pituitary gland, sPTHrP staining was detected in cells of the pi bordering the pn and these sPTHrP-positive cells were identified as SL-producing cells. This was confirmed in earlier studies, by Rand-Weaver et al. (1991) and Kaneko et al. (1993), who found SL staining in PAS-positive cells that border the pn, in several teleosts. Our data confirm an earlier claim by Ingleton et al. (1998) who reported that in sea bream, sPTHrP and SL are both located in the PAS-positive cells and that some cells contained both sPTHrP and SL. SL is a hormone from the *prl* gene family and is structurally related to both PRL and growth hormone. Kakizawa et al. (1993) studied SL plasma levels and *sl* mRNA expression in rainbow trout and suggested a role for SL in calcium balance and an increased hormone turnover rate at low calcium levels. Changes in SL plasma levels and pituitary gland mRNA expression at low ambient calcium appear only after several days (Kakizawa et al., 1993), which makes short-term effects of SL on calcium balance unlikely. However, the activity of SL-producing cells may be affected indirectly, possibly by the action of PTHrP. Our data show sPTHrP-immunostaining in sea bream pituitary gland SL-producing cells and activation of *pthrp* mRNA production in the pituitary gland 3 h after transfer from SW to DSW. It could very well be that in cells co-expressing *pthrp* and *sl*, the *pthrp* up-regulation precedes that of *sl* and therefore the activity of SL in the pituitary gland. Interestingly, the pituitary gland sPTHrP-producing cells co-localise with a sub-population of SL-producing cells, the SLα cells as observed in zebrafish (Zhu et al., 2004).

mRNA expression

Expression of *pthrp* and *pth1r* mRNA was found in all tissues examined indicative of an auto-/para- or intracrine function of sPTHrP. However, circulating plasma PTHrP levels in teleosts and elasmobranches, as well as immunostaining (Trivett et al., 1999) and mRNA expression (Hang et al., 2005) for *pthrp* in pituitary glands have been established, pointing to an endocrine function for PTHrP as well.

Down-regulation of *pthrp* and *pth1r* mRNA in the pituitary gland was established after 3 h of calcium constraint and maintained reduced after at least 30 days of calcium constraint. Both a rapid activation of pituitary gland sPTHrP production and a long-term involvement of sPTHrP in the

adaptation to hypocalcic media seem required to maintain calcium balance at hypocalcic conditions. This suggestion is supported by the correlations that were found between plasma Ca^{2+} and pituitary gland *pthrp* mRNA expression and between plasma Ca^{2+} and plasma sPTHrP protein. However, the down-regulation of *pthrp* and *pth1r* mRNA in the pituitary gland was not accompanied by a change in plasma sPTHrP level, which had only slightly decreased in the group that was held at DSW and fed a Ca– diet for 30 days. The adaptive response to calcium constraint results in a reduced metabolic clearance of sPTHrP from the plasma (in contrast with the reported action for SL), with down-regulated mRNA expression in the pituitary gland and unaltered plasma sPTHrP levels. This suggests a differential regulation of release of sPTHrP and SL in cells that co-express these proteins or, alternatively, a differential regulation of the two pituitary SL cell populations, recently reported by Zhu et al. (2004).

The five- to eight-fold up-regulated peripheral *pthrp* and *pth1r* mRNA levels in gills after 30 days exposure to low calcium may reflect an adaptive response, possibly as a result of a decreased environmental calcium concentration or a reduced sPTHrP metabolic clearance from the plasma by auto-regulatory feedback of sPTHrP on its own secretion. In earlier studies on mammals, Fujimi et al. (1991) suggested that PTH(1-34) directly inhibits PTH secretion in parathyroid cells In contrast, Lewin et al. (2003) hypothesized that PTH has a positive auto-feedback on its own secretion under hypocalcic conditions in rats.

Flanagan et al. (2000) showed PTHrP staining in the chloride cells of gills in teleosts and identified these cells as the principal location for PTHrP in gill tissue. The increase in chloride cell density in diluted sea water conditions could explain the increase in *pthrp* mRNA that was found in gills. Chloride cells are the site of branchial Ca^{2+} uptake in gills and also contain the CaSR. However, expression of *casr* mRNA was unaffected in gills after calcium restraint. The CaSR is regulated and equipped to respond to the blood Ca^{2+} level. Fluctuations as small as 0.2 mmol l^{-1} can be sensed (Lopez-Ilasaca et al., 1997), which enables the fish to tightly regulate the blood Ca^{2+} concentration. In the present study, the plasma Ca^{2+} level was remarkably constant and maintained within a maximal range of 0.2 mmol l^{-1} difference when compared with the controls and therefore up-regulation of *casr* mRNA may not be relevant. In the pituitary gland, a significant down-regulation of

casr mRNA was observed after both short- and long-term calcium constraint that indicates that the need for calcium-controlled processes had decreased or reflecting a need for desensitivisation to Ca^{2+} signals. Flanagan et al. (2002) located immunostaining for the CaSR in cells bordering the pn in both the intermediate and distal pituitary lobes of the sea bream and suggested a possible feedback between fibres from hypothalamic nuclei and pituitary factors affected by calcium. This is supported in this study by the localisation of sPTHrP in cells near the pn, which possibly control the up-regulation of *pthrp* gene expression in the gills.

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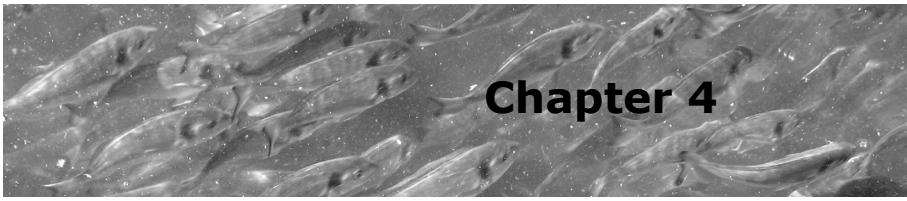

Chapter 4

PTHrP regulation and calcium balance in sea bream (*Sparus auratus* L.) fed a vitamin D-deficient diet

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Abstract

Gilthead sea bream (Sparus auratus L.) were fed a vitamin D-deficient diet for 22 weeks. Growth rate, whole body mineral pools and calcium balance were determined. Plasma parathyroid hormone related protein (PTHrP) and calcitriol levels were assessed. Expression of mRNA for pthrp and pth1r was quantified in gills and hypophysis. Fish on vitamin D-deficient diet (D- fish) showed reduced growth and lower calcium turnover (calcium influx, efflux and accumulation rates decreased) and unaltered plasma calcium levels. Plasma calcitriol levels became undetectable, PTHrP levels decreased in the D- fish. In controls, a significant increase in plasma PTHrP level over time was seen, i.e. it increased with body mass. Relationships were found between plasma PTHrP and the whole body pools of calcium, phosphorus and magnesium, indicative of a role for PTHrP in bone development. Expression of pthrp and pth1r mRNA was down-regulated in the hypophysis of D- fish, whereas in gill tissue, pthrp and pth1r mRNA was up-regulated. We conclude that lower pthrp mRNA expression and plasma values in D- fish reflect lower turnover of PTHrP under conditions of hampered growth; up-regulation of pthrp mRNA in gills indicate compensatory paracrine activity of PTHrP during calcitriol deficiency to guarantee well-regulated branchial calcium uptake. This is the first report to document a relation between PTHrP and calcitriol in fish.

Introduction

In teleost fish, as in higher vertebrates, calcium plays a key role in a variety of biochemical and physiological processes. Fish have access to and use the essentially infinite sources of calcium in the water (through branchial uptake mechanisms). Their skeleton and dermal scales, important for shape, armour, structure and muscle attachment, serve as an important internal reservoir for calcium and phosphorus. In teleosts, about 99% of the total calcium pool is incorporated into the skeleton and dermal scales in the form of calcium phosphates and to a lesser extent as calcium carbonate (Flik et al., 1986).

In fish blood, the total calcium concentration is 2-3 mmol l^{-1}, of which the physiologically important ionic fraction is about 1.25 mmol l^{-1} (Hanssen et al., 1991). The blood calcium level may vary among species and within species that are euryhaline, but is always strictly regulated at varying set points and in accordance with environmental calcium availability by hyper- and hypocalcemic hormones. Of these hormones, stanniocalcin, produced by the corpuscles of Stannius, is the dominant hypocalcemic (in fact anti-hypercalcemic) hormone. Stanniocalcin secretion is stimulated by increased plasma calcium concentrations and inhibits calcium influx from the environment via the gills and intestine (Verbost et al., 1993).

Parathyroid hormone related protein (PTHrP) is an important hypercalcemic factor in early vertebrates and is present in the cartilagenous sharks and rays (Ingleton et al., 1995; Trivett et al., 1999) and bony fishes (Danks et al., 1993; Ingleton 2002). Three different receptors for PTHrP have been identified in zebrafish (*Danio rerio*, Hamilton 1822; Rubin and Jüppner, 1999) and more recently, in sea bream (*Sparus auratus* L.; Rotllant et al., 2005), of which PTH1R is the most common.

In sea bream, PTHrP has been detected in the plasma and several tissues, using a radioimmunoassay with antisera to the human peptide (Devlin et al., 1996) and more recently, with a homologous radioimmunoassay (Rotllant et al., 2003). PTHrP is involved in a number of physiological functions in this fish, such as bone and scale development (Martin et al., 1997; Redruello et al., 2005), vitellogenesis (Guerreiro et al., 2002; Bevelander et al., 2006) and calcium regulation (Guerreiro et al., 2001; Abbink et al., 2004; 2006; Fuentes et al., 2006).

Vitamin D is not synthesized by fish through photochemical processes in the skin and therefore, the diet is assumed the key source of vitamin D. Teleosts have large stores of vitamin D in their liver, and this precursor can be converted into hydroxylated metabolites (Graff et al., 1999). The active metabolite of vitamin D, $1,25(OH)_2D_3$ or calcitriol, is a steroid hormone that exerts its effects through a high affinity vitamin D_3-receptor (VDR; DeLuca and Zierold 1998; our personal observations on salmon, trout and carp). Calcitriol plays a role in fish calcium metabolism by stimulation of intestinal calcium absorption (Swarup et al., 1991; Sundell et al., 1993) and is a key factor in bone formation (Haga et al., 2004); the effects of calcitriol can be considered hypercalcemic, in mammals and fish alike.

Sundell et al. (1992) demonstrated increased calcium absorption after calcitriol administration and localized calcitriol receptors in several calcium regulating tissues (gill, intestine) in Atlantic cod (*Gadus morhua* L.). Moreover, vitamin D and its metabolites, including calcitriol, have been found in plasma of various fish species (Takeuchi et al., 1991; Horvli et al., 1998).

In this study we investigated the influence of feeding juvenile gilthead sea bream a vitamin D-deficient diet for prolonged time on the regulating role of PTHrP in maintaining the calcium balance. We hypothesized that denying fish vitamin D through a vitamin D-deficient diet would result in recruitment of hypercalcemic PTHrP to maintain calcium balance and to counteract the imminent threat of hypocalcemia. The experiments were carried out under controlled laboratory conditions and lasted for five months. Fish were weighed at the start of the experiment, after acceptance of the diet, *i.e.* two weeks later and then every 4 weeks, to follow growth performance. Fish were sampled at four-week intervals to assess whole body mineral pools, as of 6 weeks after the start of the experiments and than every 4 weeks; calcium balance (calcium fluxes) were determined at 18 weeks into the experiment when significant differences in growth were observed. Blood was collected as of 6 weeks into the experiment and used to prepare plasma for analyses of calcium (weeks 18 and 22), PTHrP (weeks 10, 14, 18, 22) and calcitriol (weeks 18 and 22) levels; mRNA expression for *pthrp* and *pth1r* were assessed by real time quantitative polymerase chain reaction (RQ-PCR; weeks 18 and 22). The

small volume of plasma available per fish forced us to make this analysis schedule.

Material and methods

Fish

Juvenile gilthead sea bream (*Sparus auratus* L.) weighing around 0.5 g were obtained from a commercial fish farm (Viveiro Vilanova, Lda., V.N. Milfontes, Portugal) and flown to The Netherlands without mortality. The fish were kept in a round 1200-L tank with aerated flow-through, a constant salinity of 34‰ and a water temperature of 23 ± 1°C. The water quality was monitored continuously for nitrogenous waste products and pH. The fish were fed daily with commercial pellets (Trouvit, Trouw, Putten, The Netherlands) at a ration of 2% of the total body mass. This ration allowed the fish to grow and did not lead to detectable nitrogenous waste build-up in the tanks. The treatment of the fish was in agreement with the Declaration of Helsinki and Dutch law concerning animal welfare, as tested by the ethical committee for animal experimentation of the Radboud University Nijmegen.

Experimental set-up

The fish were kept in control tanks for two months post transport. At the start of the experiment (t = 0), 350 fish (3.91 ± 0.69 g) were randomly selected from stock and transferred to two 500L tanks (175 fish in each tank), with identical water conditions as in the stock tank. One week after transfer, the diet was changed from commercial pellets (Trouvit) to the test pellets (Hope Farms, Woerden, The Netherlands). The two diets did not differ in energetic value; the phosphorus content of the diets was 5.8 g Kg^{-1}, the calcium content 11.0 g Kg^{-1}. The only difference between the diets was the vitamin D and D3 contents of the sufficient diet, which were 1 IU g^{-1}.

Within a week, the fish accepted the new diet and showed normal appetite, *i.e.* the fish ate all the food provided (t = 2). Indeed, the fish continued to grow during the change in diet (time period t = 0 to t = 2 weeks of the experiment; Fig. 1). The experimental fish were always fed first and the controls received an equivalent amount of food as taken by the experimental fish. The fish were given a vitamin D-sufficient diet (controls; D+ fish) or a vitamin D-deficient diet (D- fish). Information on the diet is available on request.

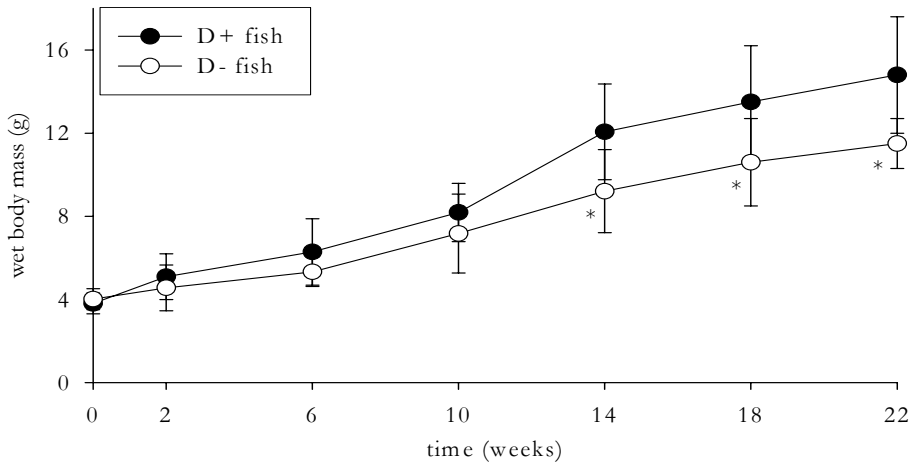

Figure 1. *Growth of the fish during the 22 weeks of the experiment. The fish of both groups continued to grow throughout the whole experiment. As of 14 weeks of feeding the vitamin D-deficient diet, a significantly reduced body mass was found in the D- fish. Asterisks represent significant difference from control group (P<0.05).*

The experiment had five sampling points for various analyses, starting at 6 weeks into the experiment (t = 6; four weeks after acceptance of the new diet) with four weeks intervals. The day before sampling, the fish were not fed. To collect blood, fish were deeply anaesthetised with 2-phenoxyethanol (1:500; Sigma-Aldrich, St. Louis, MO, USA); the caudal vessels were punctured with a 23-G needle fitted to a tuberculine syringe, rinsed with sodium heparin (Leo Pharma, Weesp, The Netherlands; 5000 U ml^{-1}) to avoid blood clotting. Next, the fish was killed by spinal transection. Bone and muscle samples were taken to assess whole body mineral pools; plasma was analysed for calcium, PTHrP and calcitriol levels. Selected tissue sub-samples were taken to assess mRNA expression levels for *pthrp* and *pth1r*.

Drinking rate (*DR*) and calcium influx (F_{in}^{Ca2+}) were evaluated, using ^{51}Cr-EDTA (25.75 GBq mg^{-1}; Perkin-Elmer, Boston, MA, USA) and $^{45}CaCl_2$ (0.55 GBq mg^{-1}; Perkin-Elmer) respectively, according to procedures that have been validated extensively (Flik et al., 1986); also for sea bream (Guerreiro et al., 2004).

Whole-body mineral content

The body mass of the fish was determined upon transfer from stock tank to test tank (t = 0), after acclimatisation to the experimental diets (t = 2, after two weeks) and at the subsequent five sampling points (t = 6, after 6 weeks, t = 10, after 10 weeks *etc.*); the experiment lasted a total period of 22 weeks.

At every sampling point, 15 fish of each group were euthanized with 2-phenoxyethanol (1:500), were freeze-dried until constant weight (DW) and dissolved in concentrated nitric acid (70%; 3 ml g^{-1} DW; Sigma-Aldrich) for whole body mineral analyses. The nitric acid digests were diluted 500x with demineralised water and analysed for calcium, phosphorus and magnesium, using inductively coupled plasma atomic emission spectrophotometry (ICP-AES, Plasma IL200; Thermo Electron, MA, USA). From these analyses, whole body pools were calculated. Mineral content was expressed in μmol g^{-1} DW, based on the fish DW and the total digest volume.

Plasma parameters

Blood was taken as indicated above from 15 fish of both groups at sampling points t = 6 to t = 22. The collected blood was centrifuged at 13.600g for 10 min and the plasma so obtained was stored at -20°C. Plasma Ca^{2+}, Na^{+}, K^{+}, glucose, lactate (mmol l^{-1}) and pH were measured using a Stat Profile pHOx plus analyser (Nova Biomedical, Waltham, MA, USA) and plasma total calcium (mmol l^{-1}) was measured using ICP-AES. The plasma PTHrP level (nmol l^{-1}) was measured with a homologous radioimmunoassay according to Rotllant et al. (2003) and the plasma calcitriol level (pmol l^{-1}) was measured according to van Hoof et al. (1993).

Table 1. *Plasma minerals, glucose, lactate (in mmol l^{-1}) and pH in sea bream were unaffected in the vitamin D deficient fish after 22 weeks of feeding the diet (n = 8; values are given as mean ± S.D.).*

	total Ca	Ca^{2+}	Na^{+}	K^{+}	Glucose	Lactate	pH
D+ fish	2.3 ± 0.7	1.10 ± 0.21	159 ± 9	4.2 ± 0.5	7.7 ± 3.6	3.1 ± 1.1	7.66 ± 0.04
D- fish	2.2 ± 0.4	0.99 ± 0.15	155 ± 4	4.4 ± 0.4	5.2 ± 3.2	3.3 ± 2.4	7.64 ± 0.04

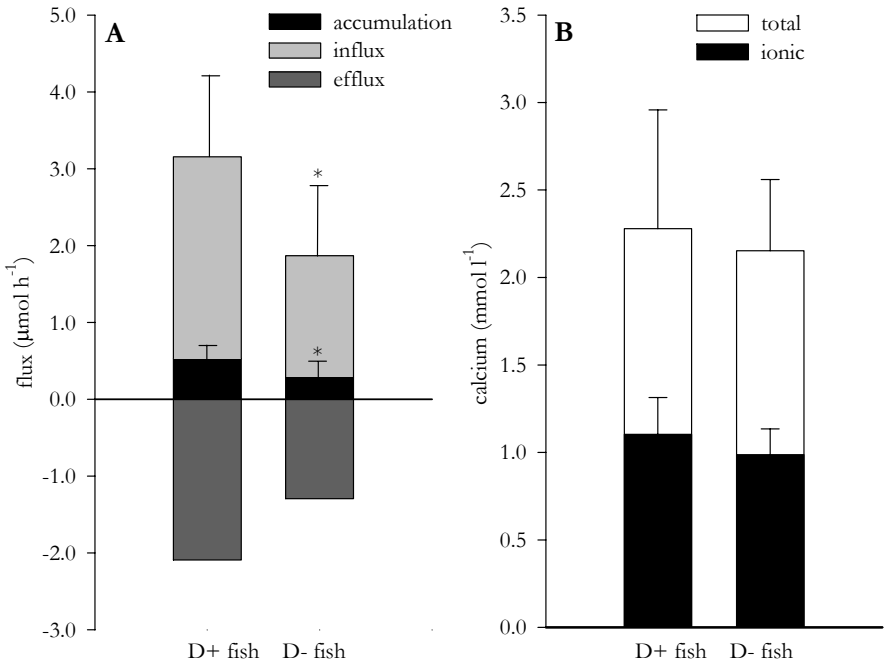

Figure 2. *Calcium balance shows the reduced calcium turnover, with a decreased calcium influx, efflux and accumulation rate in the D- fish (**Fig. 2A**) and the unaltered plasma calcium levels in the D- fish when compared with the controls (**Fig. 2B**).*

Drinking and calcium influx

After 18 weeks into the experiment (t = 18), 20 fish from each group were randomly divided and placed into identical vessels, with 10 fish per vessel to determine the drinking rate and the calcium influx according to earlier described procedures (Flik et al., 1985; Abbink et al., 2006).

pthrp and pth1r mRNA expression

At 18 (t = 18) and 22 weeks (t = 22) into the experiment, tissue samples from gill and the pituitary gland were taken from eight fish from the D+ and D- group. The small size of the fish and consequently of the pituitary gland did not allow us to take samples for RQ-PCR analysis at time points t = 6, t = 10 and t = 14; mRNA expression was successfully analysed in individual fish on the subsets of samples taken at t = 18 and t = 22 weeks. RQ-PCR was used to

quantitate mRNA expression levels for *pthrp* and *pth1r* in these tissue samples according to Hang et al. (2005), using the house-keeping gene β-actin as an endogenous control.

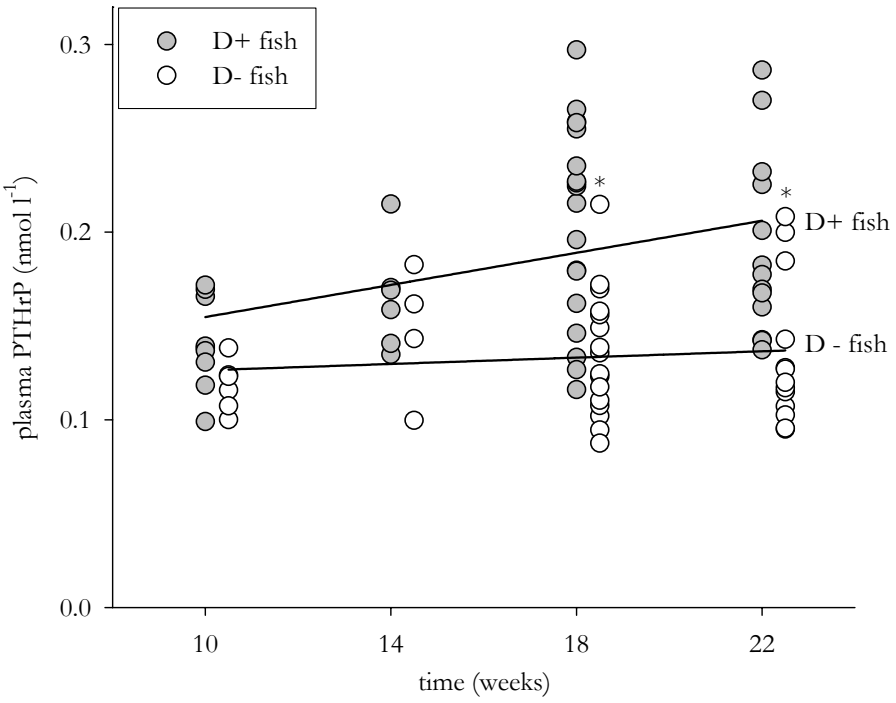

Figure 3. *The fish that were fed a vitamin D-deficient diet show a decreased plasma PTHrP level when compared with the control fish. Further on, a significant correlation between plasma PTHrP and body weight is found in the D+ fish ($R^2 = 0.14$, $N = 46$, $p < 0.05$). In the D- fish, this correlation is not present ($R^2 = 0.01$, $N = 38$, $p > 0.05$).*

Statistical analysis

All data are expressed as means ± standard deviation (S.D.). Differences among groups were assessed by ANOVA. Significance of differences was assessed by parametric (Student's *t*-test) or non-parametric (Mann Whitney *U*-test) tests where appropriate and $P < 0.05$ was taken as fiducial limit.

Results

Calcium balance

No mortality was observed during the experiment; a lower body mass of the D- fish when compared with the D+ fish occurred as of 14 weeks of feeding the deficient diet (Fig. 1). Plasma minerals, glucose, lactate and pH were unaffected in the vitamin D-deficient fish after 22 weeks of feeding the diet (Table 1). The growth related calcium accumulation rate (Fig. 2A) is lower in the D- fish (0.29 ± 0.29 μmol h^{-1}) when compared with the D+ fish (0.53 ± 0.20 μmol h^{-1}). Unidirectional calcium influx, $F_{in}Ca^{2+}$, was 2.62 ± 1.51 μmol h^{-1} in the D+ fish and had decreased to 1.58 ± 1.14 μmol h^{-1} in the D- fish. The calcium efflux (calculated as the difference between calcium the influx minus net accumulation rate) was 2.09 μmol h^{-1} in the D+ fish and was 1.30 μmol h^{-1} in the D- fish. However, the ratio between calcium influx, efflux and accumulation remained constant and the plasma total and ionic calcium concentrations were not affected by the vitamin D-deficient diet (Fig. 2B).

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Feeding the fish a vitamin D-deficient diet decreased the calcitriol concentration to a level below the assay's detection limit (<175 pmol l^{-1}) after 18 and 22 weeks of feeding the diet (n = 8). In the controls, plasma calcitriol remained at a constant level throughout the experimental period (228 ± 35 pmol l^{-1} after 18 weeks and 245 ± 58 pmol l^{-1} after 22 weeks; n = 8). In addition, strongly decreased plasma PTHrP levels were found in the D- fish after 18 and 22 weeks of feeding the diet (Fig 3; 18 weeks: D+ fish: 0.21 ± 0.05 nmol l^{-1}, n = 15; D- fish: 0.13 ± 0.03 nmol l^{-1}, n = 15, $P < 0.001$ and 22 weeks: D+ fish: 0.19 ± 0.04 nmol l^{-1}, n = 13; D- fish: 0.13 ± 0.04 nmol l^{-1}, n = 13, $P < 0.001$).

In the control fish, body weight and plasma PTHrP were positively correlated (Fig 3; $R^2 = 0.14$; n = 42; $P < 0.01$), as were the whole body pools of major bone minerals and PTHrP (calcium: Fig. 5A; $R^2 = 0.69$; n = 4; $P < 0.05$; phosphorus: Fig. 5B; $R^2 = 0.75$; n = 4; $P < 0.05$ and magnesium: Fig. 5C; $R^2 = 0.68$; n = 4; $P < 0.05$). In the D- fish, all these relationships were abolished. Relationships between body mass of the fish and whole body pools for calcium, phosphorus and magnesium were unaffected in the D- fish (data not shown).

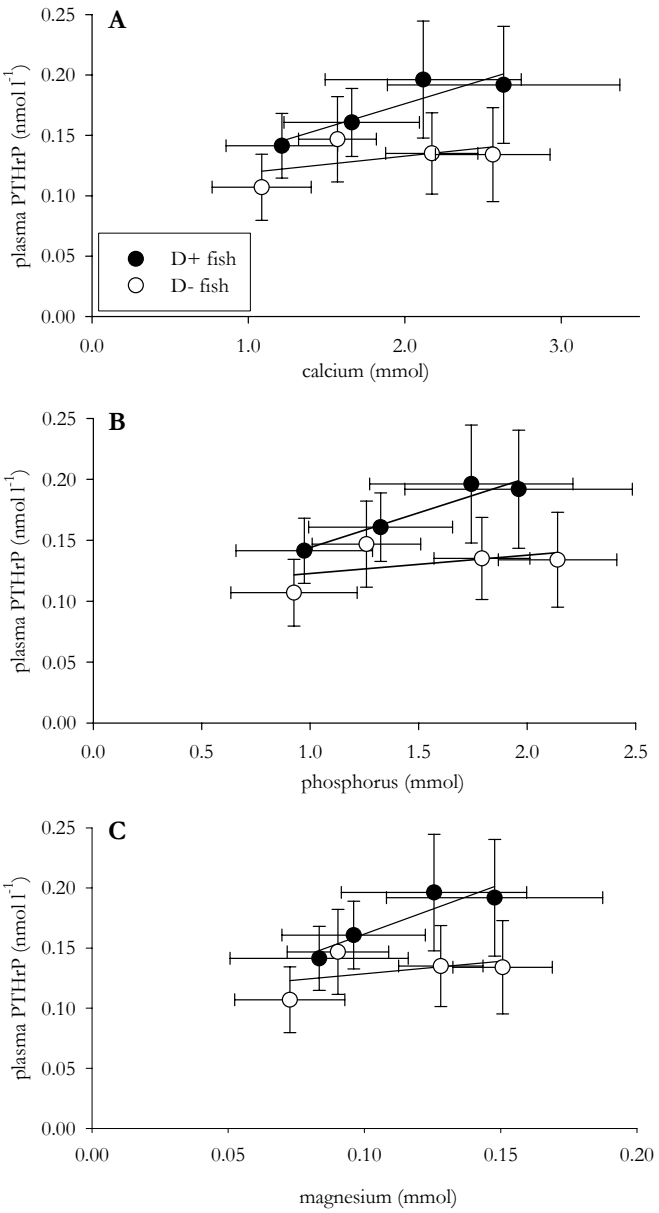

Figure 4. *Correlations between plasma PTHrP and whole body calcium, phosphorus and magnesium pools in the D+ fish (Fig. 5A: calcium; $R^2 = 0.69$, $N = 5$, $p < 0.05$; Fig. 5B; phosphorus: $R^2 = 0.75$, $N = 5$, $p < 0.05$; Fig. 5C: magnesium; $R^2 = 0.68$; $N = 5$; $p < 0.05$). In the D- fish, these relations were absent (Fig. 5A: calcium; $R^2 = 0.42$, $N = 5$, $p > 0.05$; Fig. 5B: phosphorus; $R^2 = 0.38$, $N = 5$, $p > 0.05$; Fig. 5C: magnesium; $R^2 = 0.31$; $N = 5$; $p > 0.05$).*

The mRNA levels for *pthrp* and *pth1r* in the pituitary gland and gills did not show any differences after 18 and 22 weeks of feeding the diet and the data were therefore pooled. In the pituitary gland, mRNA expression was down-regulated in the D- fish when compared with the control group (Fig. 6), whereas in gills, an up-regulation for *pthrp* and *pth1r* mRNA was found in the D- fish compared to controls.

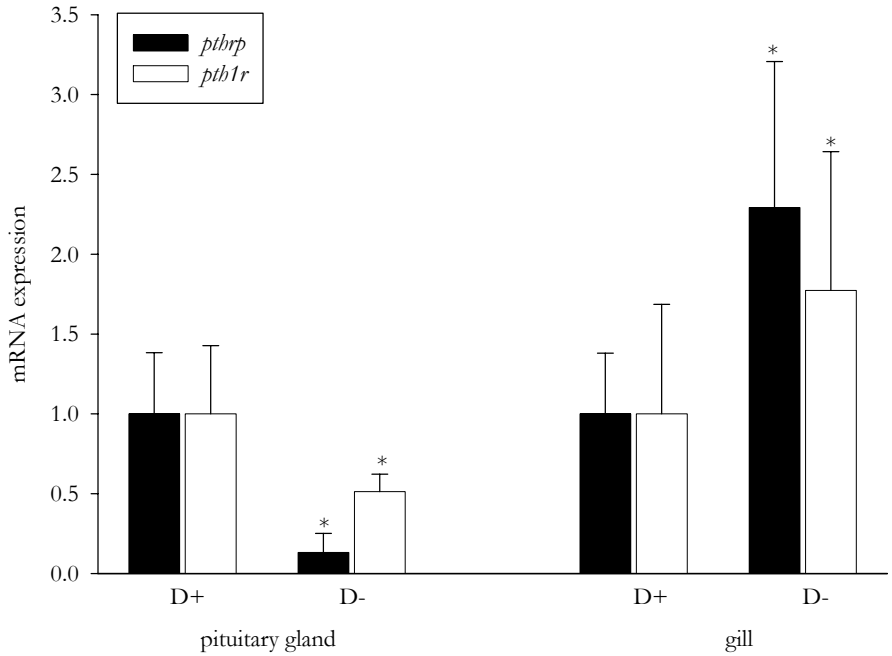

Figure 5. *Expression for pthrp and pth1r mRNA is down-regulated in the pituitary gland of the D- fish when compared with the D+ fish. In contrast, gill tissue showed an up-regulated mRNA expression level for pthrp and pth1r in response to the vitamin D-deficient diet.*

Discussion

We hypothesized that fish on a vitamin D-deficient diet would recruit hypercalcemic PTHrP for calcium balance and to counteract the imminent threat of hypocalcemia. This hypothesis was, to our surprise, only partly confirmed. Under long term vitamin D constraint sea bream show a lower

growth rate due to decreased calcium turnover (yet, the fish keep their calcium balance) and decreased plasma PTHrP and calcitriol levels. The positive correlations between plasma PTHrP and bone minerals (calcium, phosphorus and magnesium) became less prominent. Expression of *pthrp* and *pth1r* mRNA was down-regulated in the pituitary gland and up-regulated in gill tissue.

As suggested earlier (Abbink et al., 2006) we have evidence for an independent branchial PTHrP regulatory system, acting in a paracrine fashion and apart from an endocrine pituitary source of PTHrP. The branchial chloride cell expresses PTHrP (Flanagan et al., 2000), is of major importance in calcium uptake in fish (Flik et al., 1995) and thus appears to be fitted with a paracrine (and/or auto- and/or intracrine) calciotropic or cell proliferation control mechanism. Such actions of PTHrP are well recognised.

Calcium balance

The absence of vitamin D in the diet slowed down bone formation and growth rate, and thus reduced the need to incorporate calcium into the skeleton and dermal scales, processes that require calcitriol (Graff et al., 2002) and PTHrP (Redruello et al., 2005; Rotllant et al., 2005), and indeed the levels of both hormones had decreased in fish kept on a vitamin D-deficient diet. An earlier study by Taveekijakarn et al. (1996) described impeded growth in response to vitamin D-deficient diet in amago salmon (*Oncorhynchus rohdurus*, Jordan and McGregor 1925). However, Graff et al. (2002) found no difference in growth rate in Atlantic salmon (*Salmo salar* L.) fed a low level vitamin D diet (0.2 mg kg^{-1}) for three months. Indeed, the low vitamin D level present in the latter diet could still suffice to guarantee growth, considering the relatively mild effects seen in our study with deficient diet; it should be noted that we did not observe any effects in the early phase of feeding the diet.

The fish of both groups continued to grow and their whole body calcium content increased, while a strict and constant relation between the total calcium, phosphorus and magnesium pools and body weight was kept, indicating well-adapted mineral handling. Under stress conditions often enhanced calcium efflux and decreased uptake mark disturbances of calcium balance (Flik et al., 1985); no such phenomena were seen in our study. Reduced growth rate and decreased accumulation rate coincided with

down-scaled calcium influx and efflux rate, *i.e.* the calcium turnover decreased but calcium balance was not disturbed. This peculiar adaptive response may be easily overlooked (little seems to change other than growth rate) but lower calcium turnover coincides with altered calciotropic activities (see next section). The lower calcium turnover was not accompanied by a decrease in plasma calcium levels, which remained constant during the experiment. Since minor deflections in plasma ionic calcium levels can lead to severe physiological disruptions, also in fish (Flik et al., 1995), plasma ionic calcium levels must be and are regulated within narrow limits. Injections of calcitriol lead to increased plasma calcium levels, in line with predicted hypercalcemic actions of calcitriol (Fenwick et al., 1984; Sundell et al., 1993). Clearly, in some of the experimental fish in this study calcitriol levels became even undetectable and PTHrP levels remained constant. The unaffected plasma ionic calcium level then would indicate PTHrP turnover had decreased, yet PTHrP activity remained sufficient to cope with the threat of hypocalcic conditions in the vitamin D-deficient fish.

Endocrinology

Two remarkable and new observations were made when the plasma PTHrP levels in the fish were analysed. First, the vitamin D-deficient diet resulted in undetectable calcitriol levels and in highly significant lower plasma PTHrP levels after 18 and 22 weeks of feeding the diet, indicating that the vitamin D-deficient diet results in adaptive responses in the plasma PTHrP level, but only after 14-18 weeks of feeding the diet. A second remarkable finding is the small, but significant tendency for plasma PTHrP to increase over time, *i.e.* it increased with the increasing mass of the fish. In earlier studies, Rotllant et al. (2003) measured plasma PTHrP in sea bream and found 2.5 ± 0.29 ng ml^{-1} (0.61 ± 0.07 nmol l^{-1}) in 100-150 g sea bream and Abbink et al. (2004) measured somewhat lower values 0.21 ± 0.06 to 0.32 ± 0.12 nmol l^{-1} in smaller, 40-60g juvenile sea bream. These levels are in line with the values found in the present study and establish once more a relation between the plasma PTHrP level and the increasing body mass of the fish. In accordance, Abbink et al. (2006) showed that in juvenile sea bream the plasma PTHrP level increases with the body mass, but plateaus with increasing mass of the fish; we suggested a role for PTHrP in skeletal physiology in particular in juvenile fish (where the bone compartment is relatively large) and thus a

decreasing need for hypercalcemic control with increasing body mass. The strong correlations between plasma PTHrP and the whole body content of the main minerals in bone (calcium, phosphorus and magnesium) that were found in the present study further strengthen the assumption that PTHrP is involved in skeletal calcium physiology. Taken the above mentioned aspects into account we argue that plasma PTHrP levels reflect the need for regulation of the bone compartment, which is compartment/size dependent.

Involvement of PTHrP in skeleton and scale calcium physiology was suggested earlier. Rotllant and co-workers (2005) established PTHrP involvement in calcium reabsorption from scales when the activity of tartrate-resistant acid phosphatase (TRAPC; a marker for osteoclastic activity in mammalian bone) was enhanced when cultured sea bream scales were treated with N-terminal (1-34)PTHrP. Redruello et al. (2005) measured abolished osteonectin mRNA expression in sea bream scales treated with different doses (10 and 1000 nmol l^{-1}) of (1-34)PTHrP. Osteonectin is a calcium binding glycoprotein that stimulates the mineralization process following differentiation of the osteoblastic cell lineage (Estevao et al., 2005).

The loss of correlations between plasma PTHrP and the whole body pools for calcium, phosphorus and magnesium in the D- fish point to a disturbance of the bone formation process induced by the vitamin D-deficiency. The subsequent decrease in growth rate and calcium turnover could have evoked the adaptive responses of the branchial and pituitary PTHrP systems, as presented in this study. Expression levels for *pthrp* and *pth1r* mNRA in sea bream have been described earlier by Flanagan et al. (2000) and Hang et al. (2005) and have established a widespread tissue distribution of PTHrP, mostly with a low expression level. The distribution pattern is suggestive of para-, auto- or intracrine functions of PTHrP. However, the presence of PTHrP in the pituitary gland and the (high) circulating levels (in fish indeed at concentrations of other endocrines, in the nmol l^{-1} range) point to a classic endocrine function for PTHrP, as suggested earlier by Danks et al., (1993) and Abbink et al. (2006).

Down-regulated expression levels for *pthrp* and *pth1r* in the pituitary gland suggest that the vitamin D-deficient diet with its consequences for calcium turnover may target and feedback at the pituitary gland level. This is reflected then by a decreased plasma PTHrP level, and would point to a specific calcitriol feedback on the pituitary somatolactin cells that produce

PTHrP (Ingleton et al., 1998; Abbink et al., 2006). Clearly, coinciding lower calcium turnover and lower pituitary PTHrP turnover are indicated by our data.

The up-regulation of branchial *pthrp* and *pth1r* mRNA expression correlates well with long-term adjustment of uptake-mechanisms under lower calcium turnover, and could be an adaptive response to the decrease in circulating PTHrP. Binding of plasma PTHrP to its receptor in the gills (that comprise a very large volume in the fish), we speculate, may contribute -significantly- to clearance of PTHrP from the plasma and this could explain, at least partly, the lower PTHrP level in the plasma as observed after long term feeding the vitamin D-deficient diet. The enhanced levels of receptor mRNA further add to this assumption. Moreover, the decline in pituitary gland *pthrp* mRNA expression will contribute to lower plasma PTHrP levels in the D- fish. Our results on differential effects of vitamin D-deficiency towards PTHrP and PTH1R in pituitary gland and gills resemble similar findings in rats where vitamin D-deficiency increased PTHrP mRNA in keratinocytes and decreased in fibroblasts and kidney cells; the receptor mRNA increased in keratinocytes and kidney, but not in fibroblasts (Errazahi et al., 2004). Apparently, such differential links between the vitamin D system and the PTHrP system (Xie et al., 1996) are universal among vertebrates.

In line with an earlier study where juvenile sea bream were given limited access to calcium (Abbink et al., 2006), the present study shows similar responses from the branchial and pituitary gland PTHrP system and is a further indication that the auto- or paracrine branchial PTHrP system acts independently from the endocrine pituitary gland PTHrP system.

This study shows that vitamin D-deficiency in fish decreases plasma calcitriol and this decrease triggers an adjustment of the pituitary and branchial PTHrP systems to counteract the imminent threat of hypocalcemia. This is the first study to reveal such relation between these two hypercalcemic hormones in fish and we speculate that the bone formation, which is calcitriol dependent, is pivotal in this relation.

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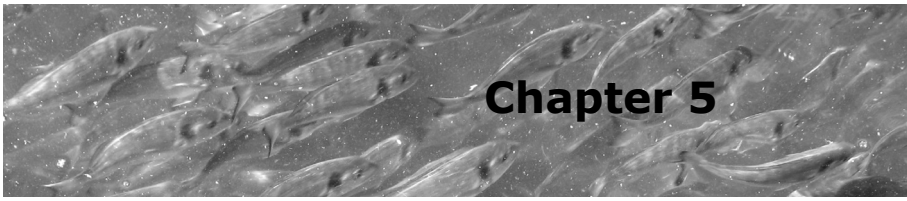

Chapter 5

Melatonin synthesis under calcium constraint in gilthead sea bream (*Sparus auratus* L.)

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General and Comparative Endocrinology doi: 10.1016/j.ygcen.2007.03.002

Abstract

Brain or blood plasma melatonin was analysed as a measure for pineal melatonin production in sea bream. Access to calcium was limited by diluting the seawater to 2.5‰ and removing calcium from the diet or by prolonged feeding of vitamin D-deficient diet. Interactions and relations between melatonin and calcium balance and the hypercalcemic endocrines PTHrP and calcitriol were assessed. Restricting calcium availability in both water and diet had no effect on plasma melatonin, but when calcium was low in the water or absent from food, increased and decreased plasma melatonin was observed, respectively. Fish on a vitamin D-deficient diet (D- fish) showed decreased plasma calcitriol levels and remained normocalcemic. Decreased brain melatonin was found at all sampling times (10-22 weeks) in the D- fish compared to the controls. A positive correlation between plasma Ca^{2+} and plasma melatonin was found ($R^2 = 0.19$; $N = 41$; $P < 0.01$) and brain melatonin was negatively correlated with plasma PTHrP ($R^2 = 0.78$; $N = 4$; $P < 0.05$). The positive correlation between plasma levels of melatonin and Ca^{2+} provides evidence that melatonin synthesis is influenced by plasma Ca^{2+}. The decreased melatonin production in the D- fish points to direct or indirect involvement of calcitriol in melatonin synthesis by the pineal organ in teleosts. The hypercalcemic factors PTHrP and calcitriol appeared to be negatively correlated with melatonin and this substantiates an involvement of melatonin in modulating the endocrine response to cope with hypocalcemia. It further points to the importance of Ca^{2+} in melatonin physiology.

Introduction

Melatonin (N-acetyl-5methoxytryptamine) is a product of tryptophan metabolism in the pineal gland and retina in all classes of vertebrates. Melatonin synthesis shows a circadian rhythm in vertebrates, including fishes, with synthesis increased during darkness and decreased during the light period (Ekström and Meissl, 1997). The rhythm of melatonin biosynthesis results from variations in activity of arylalkylamine N-acetyltransferase, the light-sensitive, key enzyme in melatonin production (Liu and Borjigin, 2005). The pineal gland does not store melatonin and therefore, levels of melatonin assayed in plasma or brain extracts directly reflect synthetic activity of the pineal gland (Kulczykowska, 2002).

The past decades have provided a plethora of data on physiological parameters that are linked to melatonin activity (Davis, 1997; Dubocovich and Markowska, 2005) that is pivotal in circadian, as well as circannual biorhythms (Meissl and Brandstätter, 1992; Reiter, 1993; Vera et al., 2006). In Atlantic salmon, *Salmo salar*, melatonin was shown to be involved in early development and control of the timing of parr-smolt transformation (Porter et al., 1998). Melatonin *per se* decreases tartrate-resistant acid phosphatase and alkaline phosphatase activities in cultured goldfish (*Carassius auratus*) scales and counteracts stimulatory effects of estradiol on these enzymes (Suzuki and Hattori, 2002). These phosphatases are the markers of choice for osteoclastic and osteoblastic activity and it thus follows that melatonin influences calcium physiology of scales (and bone) in fish (Fjelldal et al., 2004).

Many aspects of pinealocyte activity are under some control of (plasma) calcium activity and therefore, pineal function often relates directly or indirectly to calcium metabolism (Morton and Reiter, 1991). Indeed, in two fishes, rainbow trout (*Oncorhynchus mykiss*) and summer flounder (*Paralichthys dentatus*), melatonin synthesis capacity appears to be positively correlated to plasma free calcium levels (Kroeber et al., 2000; Gozdowska et al., 2003) and this relation between plasma calcium and melatonin activity warranted the research presented here. We reasoned that an analysis of plasma calcium levels and of calciotropic hormone activities would shed better light on the relation between calcemic conditions and melatonin

activity, considering the strict ionic calcemic control in fish (as in all vertebrates).

Fish have essentially unlimited access to calcium in their environment (water and diet; external calcium sources); in addition, their skeleton and dermal scales represent internal calcium sources (Flik et al., 1986). Physiological processes, such as vitellogenesis, that demand sumptuous amounts of calcium, or variations in environmental calcium availability (*e.g.* migration into soft water), require a swift calcemic endocrine system to keep plasma calcium balanced. Plasma Ca^{2+} is the physiologically important fraction in (fish) blood (Hanssen et al., 1991) and this fraction in particular is regulated within narrow limits, as even minor deviation of set point may evoke (severe) stress (Flik et al., 1995).

Calcium regulation in fishes involves the anti-hypercalcemic stanniocalcin (the hormone inhibits calcium influx from the water via the gills and by doing so exerts hypocalcemic effects; Verbost et al., 1993). It has long been thought that fish lack typical hypercalcemic endocrine factors, as antihypercalcemic control by stanniocalcin seemed to suffice in explaining calcemic control (Wagner et al., 1998). Indeed, only very recently the genes for parathyroid hormone (PTH), which is the dominant hypercalcemic factor for terrestrial vertebrates, were found in fish (Danks et al., 2003). However, earlier, fish were shown to express genes for parathyroid hormone related protein (PTHrP; Power et al, 2000; Flanagan et al, 2000; Canario et al., 2006). PTHrP behaves in fish as a hypercalcemic hormone and appears key in fish calcium physiology (Ingleton et al., 2002; Trivett et al, 2001). Recently, we established a strict relationship between PTHrP levels in plasma and plasma Ca^{2+} in juvenile sea bream (Abbink et al., 2006). PTHrP is involved in both the regulation of calcium uptake from the environment (Guerreiro et al., 2001) and regulates calcium resorption from scales (Rotllant et al., 2005). In addition to PTHrP, calcitriol ($1.25[OH]_2D_3$) exerts hypercalcemic effects in fish; it is the active metabolite of vitamin D that plays an important role in bone formation (Haga et al., 2004) and it stimulates intestinal calcium absorption (Swarup et al., 1991). Sundell et al. (1993) demonstrated calcitriol receptors in several calcium regulating tissues (gill, intestine, kidney) in Atlantic cod (*Gadus morhua*) and demonstrated increased calcium absorption after calcitriol administration, in line with hypercalcemic function. We reasoned that feeding our fish a vitamin D-deficient diet for prolonged times

should compromise their calcium physiology and thus we analysed such fish in this study.

Juvenile sea bream were limited for at least three weeks in their calcium access by feeding a calcium deficient diet, decreasing water calcium content, or both. The water calcium content was decreased by dilution of the seawater (34‰ to 2.5‰ salinity) and by doing so, the water calcium concentration decreased from 10 mmol l^{-1} to 0.7 mmol l^{-1} (Abbink et al., 2004).

Indeed, compared to untreated control fish in seawater, all experimental groups in these experiments show slightly elevated cortisol levels, although we discussed that these rises were very mild and considered still within the limits of values for non-stress situations (Abbink et al., 2004). We realise ourselves that even mild elevations of cortisol may affect neuroendocrine regulatory systems including the melatonin system (Larson et al., 2004). However, as will be shown in this paper the melatonin response to the treatments given does not parallel the earlier published cortisol responses.

In a second series of experiments, fish were fed a vitamin D-deficient diet for up to 22 weeks (Abbink et al., in press) and compared to controls that were fed a vitamin D-sufficient diet. The rationale behind these two experiments was to limit calcium availability, either directly (via water and diet) or indirectly (via vitamin D-deficiency) to impose an imminent hypocalcemia and activate hypercalcemic endocrines (PTHrP). The fish limited in their access to calcium in water and diet became hypocalcemic (for the Ca^{2+} fraction). The fish kept on the vitamin D-deficient diet remained normocalcemic, but calcium turnover decreased, indicated by decreased branchial in- and efflux of calcium and a lower calcium accumulation rate. Unexpectedly, in both experiments, plasma PTHrP levels remained constant or even decreased, while *pthrp* and *pth1r* (the main PTHrP receptor; Rubin and Jüppner, 1999) mRNA levels were down-regulated in the pituitary gland, results interpreted to indicate lower turnover of PTHrP.

Thus we followed these studies by further exploring the relationship between melatonin production and calcium regulation. In the present study we analysed the brain or blood plasma melatonin concentration of these fish and their controls to assess interactions/relations between melatonin and calcium balance and the hypercalcemic endocrines PTHrP and calcitriol.

Material and methods

Fish

Juvenile gilthead sea bream (*Sparus auratus*) were obtained from a commercial fish farm (Viveiro Vilanova, Lda., V.N. Milfontes, Portugal) and kept in a round 1500-L tank with an aerated flow-through system and full strength sea water (34‰ salinity; 10.5 mmol l^{-1} calcium) at 23°C and a photoperiod of 12 h light/12 h dark. The fish were fed a ration of 2% of the total body mass daily of commercial fish pellets (Trouvit, Trouw, Putten, The Netherlands). At the time of the experiments (spring-summer), the fish' body mass was between 10-40 g. The experimental set-up and sampling procedures were described recently (Abbink et al., 2004, 2007). In short, for the first series of experiments, four groups of fish were used. The control group A, exposed to full strength seawater (SW) and fed a control diet (Ca+ diet). Three experimental groups: group B; exposed to dilute sea water of 2.5‰ salinity (DSW), group C: fed a calcium deficient diet (Ca- diet) and group D: exposed to DSW and fed a Ca- diet). This experiment lasted for up to 3 weeks.

In the second series of experiments, fish kept in full strength seawater were fed a vitamin D-deficient (D- diet) or control diet (D+ diet) for up to 22 weeks and sampled every 4 weeks (N = 7-8). Upon completion of the experiments, the fish were quickly and deeply anaesthetised in 0.1% v/v 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) and after blood had been taken from the caudal vessels by puncture with a 24-G needle fitted to a tuberculin syringe, the fish were killed by spinal transection and the brain was promptly dissected. Animal handling followed the approved universitary guidelines. Plasma PTHrP level (nmol l^{-1}) was measured with a homologous radioimmunoassay according to Rotllant et al. (2003) and plasma calcitriol (pmol l^{-1}) was measured according to van Hoof et al. (1993).

Melatonin

The brains of the fish from the vitamin D experiment were snap-frozen in liquid nitrogen and stored at -70°C. Sonification of the brains was performed in 0.05 mol·l^{-1} phosphate buffer containing 0.01% thimerosal (Sigma-Aldrich). After centrifugation of the brain homogenate at 15,000g for 20 min, supernatant was collected and assayed for melatonin and total protein as reference. Protein was determined by the Lowry method with Peterson's

modification (Peterson, 1997), using a total protein kit (Sigma-Aldrich); bovine serum albumin (BSA) was used as a reference.

Melatonin concentration in plasma and brain samples was quantified by radioimmunoassay (RIA), using a total melatonin kit (IBL, Hamburg, Germany) with a certified extraction procedure. Solid phase extraction of melatonin from all samples (100µl) was carried out on an Octadecyl C_{18} Speedisk Column, 10µm (J.T. Baker, Phillipsburg, NJ, USA). Samples were eluted with methanol according to a procedure previously described for melatonin extraction from fish plasma (Kulczykowska and Iuvone, 1998). After extraction, samples were dried and then resuspended in Dulbecco's phosphate buffered saline containing 0.01% thimerosal and assayed by RIA. Samples were counted in a Wallac Wizard γ-counter (Wallac, Turku, Finland). The detection limit was 3.0 pg ml^{-1} in plasma and 3.5 pg ml^{-1} in brain extract. The intra- and interassay coefficients of variation for plasma melatonin were 8.0% and 15.0%, respectively. The intra- and interassay coefficients of variation for brain melatonin were 8.4% and 14.7%, respectively. Two different serum or brain samples and controls (available from IBL-Hamburg kit) were measured in ten replicates to determine intra-assay precision in the same assay. The inter-assay precision was determined by analysis of two different serum or brain samples and controls (available from IBL-Hamburg kit), in triplicate in three independent assays. The RIA data were validated by HPLC assay (Kulczykowska and Iuvone, 1998): randomly selected samples of brain and plasma were assayed for melatonin by both HPLC and RIA. The results obtained by either method were identical.

Statistics

Data are presented as means ± standard deviation (S.D.). For statistical analysis of the data, analysis of variance (ANOVA and two-way ANOVA) was used to assess differences among groups and Tukey's test was applied as post-hoc test, where appropriate. To determine relationships, regression and weighted non-linear regression analyses were performed; Pearson's correlation coefficient and y-intercept were determined, where appropriate. Significance of differences was accepted when $P < 0.05$.

Table 1. *Mineral analysis of plasma of sea bream fed a calcium-deficient diet (Ca-) while kept in dilute seawater (DSW), fed the Ca- diet in normal seawater or kept in DSW fed a normal diet. Values are in mmol l^{-1}, osmolarity is expressed in mOsmol kg^{-1}. Asterisks (*) represent significant difference from the control group (P < 0.05), N = 8 per group.*

Condition	Na^+	K^+	Ca total	Ca^{2+}	osmolality
Control	175 ± 12	5.4 ± 0.9	3.7 ± 0.3	1.30 ± 0.17	381 ± 17
Ca- diet and DSW	161 ± 8*	5.5 ± 1.4	3.3 ± 0.4*	1.15 ± 0.14*	358 ± 23*
Ca- diet	172 ± 7	5.2 ± 0.6	3.3 ± 0.5*	1.35 ± 0.09	373 ± 24
DSW	161 ± 10*	5.1 ± 1.1	3.3 ± 0.4*	1.32 ± 0.24	360 ± 28*

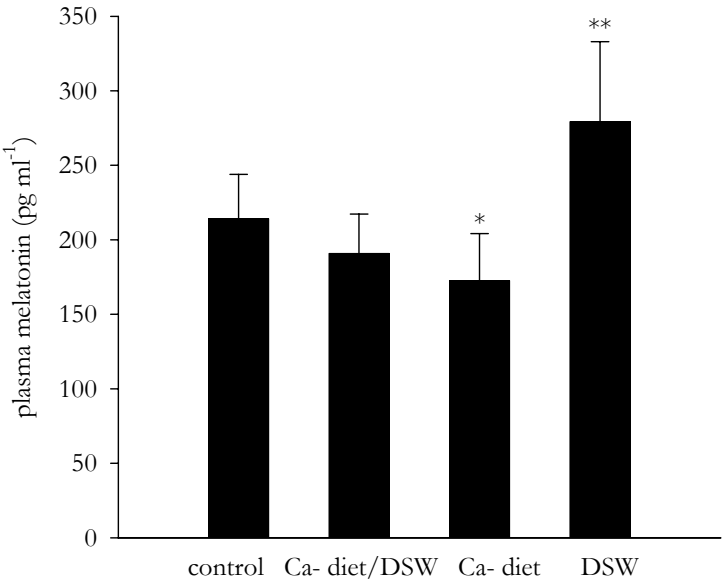

Figure 1. *Plasma melatonin levels after 3 weeks under conditions of limited calcium access. Plasma melatonin was not affected in fish kept on a Ca-deficient diet and in diluted seawater (DSW). Fish exposed to the Ca-deficient diet showed a decrease in plasma melatonin, whereas plasma melatonin was increased in fish kept in DSW. Asterisks (*) represent statistical different from the control group (* represents P < 0.05 and ** represents P < 0.01).*

Results

In fish that were restricted in their calcium access (Table 1), the total calcium level was reduced when calcium was limited in the diet (group C), whereas exposure to DSW (group D) resulted in decreased Na^+, K^+, total calcium and osmolality. Hypocalcemia (defined as decreased plasma Ca^{2+}) was only seen when calcium was restricted in both water and diet (group B).

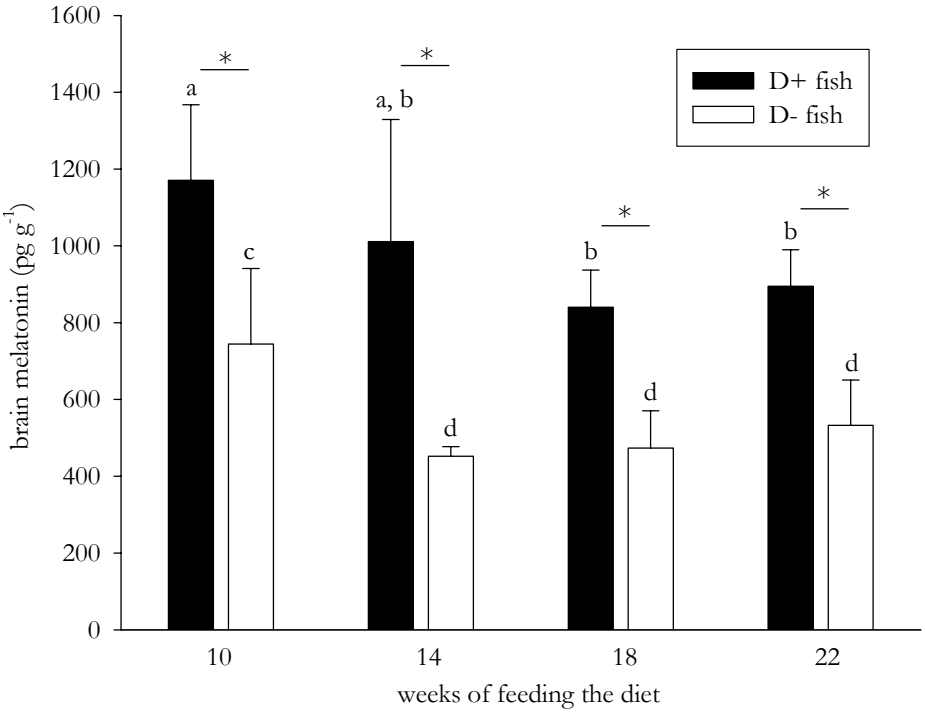

Figure 2. *In fish fed a vitamin D-deficient diet, melatonin synthesis in the brain is significantly lower compared to controls at all four time points. The reduction in melatonin was consistent and had already been established at the first sampling point. Asterisks (*) represent significant difference from accompanying control group ($P < 0.001$). The decrease in melatonin synthesis over time for the two groups is indicated by a, b for the D+ fish and c, d for the test fish ($P < 0.05$).*

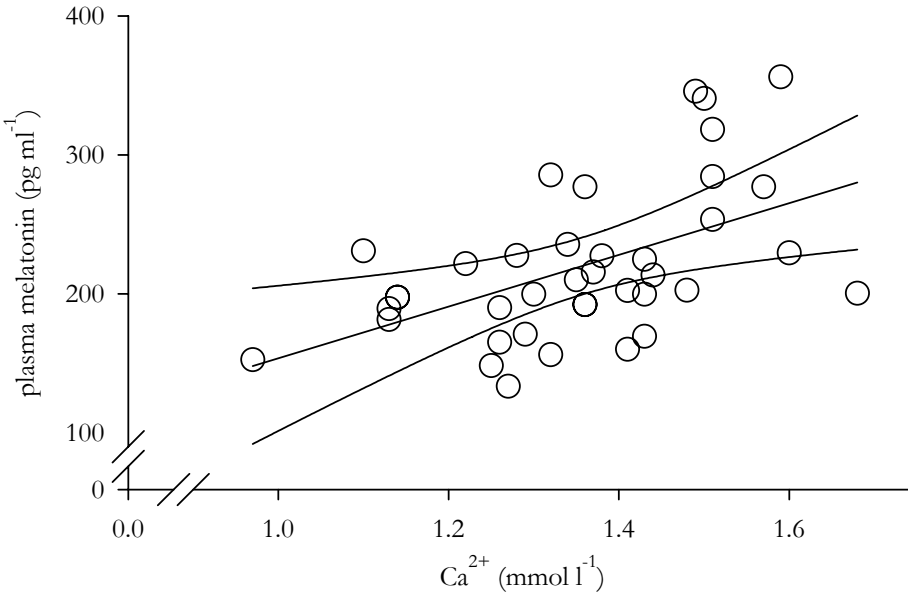

Figure 3. *Plasma melatonin correlates positively to plasma Ca^{2+} (pooled data from all fish analysed for melatonin); $R^2 = 0.19$; N = 41; $P < 0.01$. Confidence intervals (95%) are included.*

Fig. 1 shows plasma melatonin after three weeks calcium restriction. Exposure to both DSW and a Ca- diet had no effect on the plasma melatonin ($P > 0.05$). Feeding the fish (held in normal sea water) a Ca- diet decreased plasma melatonin ($F = 12.223$; $P < 0.0001$; post hoc: $P < 0.05$), whereas exposure to DSW (and fed a normal diet) resulted in an increase of plasma melatonin compared to controls ($F = 12.223$; $P < 0.001$; post hoc: $P < 0.001$).

In the D- fish, a strongly decreased brain melatonin was found at all sampling times compared to the controls (Fig. 2; F = 97.3; $P < 0.001$). The lower brain melatonin in the D- fish was established at the first sampling point, after ten weeks on the diet and was consistent throughout the subsequent experimental period. In addition, a decrease in brain melatonin was observed in time (F = 9.54; $P < 0.01$).

A positive correlation between plasma Ca^{2+} and plasma melatonin was found (Fig. 3; $R^2 = 0.19$; N = 41; $P < 0.01$).

Brain melatonin is negatively correlated with plasma PTHrP (Fig. 4; $R^2 = 0.78$; N = 4; $P < 0.05$) and this relationship was not affected by feeding

the fish a vitamin D-deficient diet (R^2 = 0.90; N = 4; P < 0.05), although plasma melatonin and PTHrP levels were lower in the latter group (D+ fish: y = -5663 ± 1717x + 1992 ± 309; D- fish: y = -6171 ± 1503x + 1363 ± 196. P = 0.96 for the slopes of the regression lines and P < 0.05 for the y-intercept).

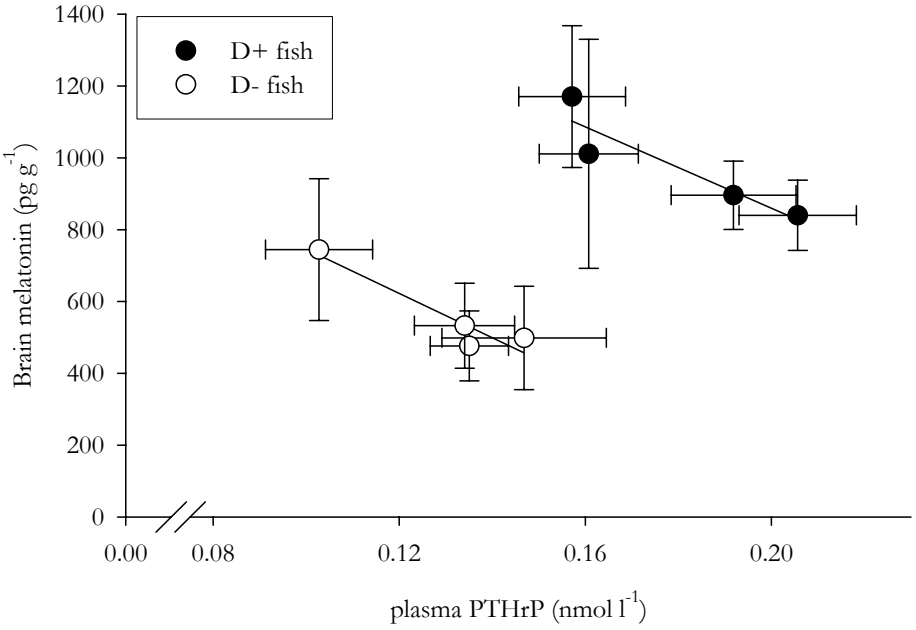

Figure 4. *Brain melatonin (production) correlates negatively to plasma PTHrP. Feeding fish a vitamin D-deficient diet does not affect this correlation, but levels of PTHrP and melatonin are decreased in concert; R^2 = 0.78; N = 4; P < 0.05 for the controls and R^2 = 0.90; N = 4; P < 0.05 for the test fish. N values represent group averages for each sampling point.*

Discussion

In the evaluation of changes in melatonin activity *in vivo* a plethora of considerations comes to mind. Melatonin controls the rhythmic adaptations to daily and seasonal cycles in fish (Bolliet et al., 1997). A variety of physiological and environmental conditions interferes with melatonin synthesis: reproduction (Mayer et al., 1997), osmoregulatory demands

(Kulczykowska 2002), photoperiod and water temperature (Garcia-Allegue et al., 2001) all affect melatonin production.
Sea bream is a protandrous fish and, being juvenile, the fish used in the present study were all sexual immature; the water temperature (23°C) and the photoperiod (12 h light/12 h dark) were kept constant and the experiments were completed in the same season. We are therefore convinced that such factors were not confounders in our experiments and that the results obtained relate mainly to calcium handling and physiology. One could argue that the effects seen in fish exposed to diluted seawater relate to altered osmoregulation or a variety of metabolic alterations which cause alterations in downstream endocrine events as a result of calcium depletion. Indeed, these faculties cannot be excluded as indicated by significant, albeit mild changes in plasma cortisol (Abbink et al., 2004) and osmolarity (this paper); yet, it should be kept in mind that diluted seawater also means a dilution of external calcium (from 10 to 0.7 mmol l^{-1}), from hypercalcic to hypocalcic conditions.

The positive relation between plasma levels of melatonin and Ca^{2+} provides further evidence that melatonin synthesis is influenced by plasma Ca^{2+} (plasma melatonin and brain melatonin reflect the synthesis capacity of the pineal gland; Kulczykowska 2002). Earlier studies (Kroeber et al., 2000; Gozdowska et al., 2003) indeed confirm the relation between plasma Ca^{2+} and the capacity of (night) melatonin production; Begay et al. (1994) observed increased melatonin synthesis in response to an increased plasma Ca^{2+} level in rainbow trout and Meissl et al. (1996) found inhibited melatonin production in a hypocalcic/low calcium medium in cultured trout pinealocytes.

Fish on a vitamin D-deficient diet (D- fish) showed decreased plasma calcitriol levels and remained normocalcemic. Growth rate was reduced, which translated in lower net calcium accumulation rate, that was confirmed by decreased branchial calcium in- and efflux (Abbink et al., in press). Feeding the fish a D- diet and the subsequent decreased calcitriol level had no visible effect on plasma Ca^{2+}, although a decreased calcium turnover was observed (Abbink et al., in press). The decrease in melatonin over time that was observed relates to the time of the year at which the experiments were conducted (spring-summer). Sokolowska et al. (2004)

showed that melatonin levels are high in early spring (March) and decrease towards the summer (July-August).

The strongly decreased melatonin production in the D- fish points to direct or indirect involvement of calcitriol in melatonin synthesis by the pineal organ in teleosts. To the best of our knowledge, there are no reports of interactions between melatonin and calcitriol in fish and reports in mammals are scarce. An interplay between melatonin and calcitriol was shown by Bizzarri et al. (2003): vitamin D (calcitriol?) enhances the synthesis of the transforming growth factor TGF-β_1, which is the most relevant negative growth regulator in breast cancer cells. Melatonin was found to increase the sensitivity of the tumor cells to vitamin D (calcitriol), thereby increasing the release of TGF-β_1 and inhibiting tumor cell growth.

The decreased melatonin synthesis in the fish fed a D- diet is in accordance with the reduced melatonin production observed in the fish fed a Ca- diet, and this suggests diet-specific effects on melatonin synthesis under calcium constraint. Melatonin produced in the intestine is the most important source of extra-pineal gland melatonin. The melatonin level in the intestinal tract is not subject to any (daily) rhythmic changes in fish (Bubenik and Pang, 1997), which indicates that the influence of plasma melatonin on intestinal melatonin physiology increases in darkness, when pineal melatonin production is up-regulated.

Rubio et al. (2004) showed that increased plasma melatonin in European sea bass (*Dicentrarchus labrax* L.), realised through orally administration in gelatin capsules, significantly reduced food intake, suggesting melatonin involvement in the process of feeding and digestion. In the present study, the indirectly (D- diet) or directly (Ca- diet) and dietary-induced calcium restraint and the subsequent calcemic endocrine action to maintain calcium balance could well have interfered with (intestinal) melatonin physiology, limiting the production of the hormone. This conclusion needs further experimentation for confirmation.

The increased melatonin production in the fish exposed to DSW is in accordance with previous studies. Kleszczyńska et al. (2006) measured plasma melatonin in sea bream adapted to different salinities and found the highest plasma melatonin in fish that were exposed to the lowest salinity. An important factor in adaptation to hypo-osmotic and hypocalcic conditions in euryhaline fishes is prolactin (PRL; Flik et al., 1994), a

hypercalcemic hormone in fish that is well known for its key role in the control of low salinity adaptation. Falcon et al. (2003) showed that melatonin reduced PRL secretion in cultured rainbow trout pituitary gland cells and provided the first evidence that melatonin modulates the secretion of PRL in teleosts. Clearly, our results indicate a positive correlation between a (presumedly) enhanced PRL activity in DSW and observed enhanced melatonin production. This in-vivo result does not corroborate the observation by Falcon et al. (2003) and suggests multivariable control; the increase in PRL in response to DSW exposure might overrule the inhibition of a PRL cell response to melatonin as observed *in vitro*.

We here argue that PTHrP is involved in the regulation of melatonin synthesis. The negative correlation between melatonin production and plasma PTHrP presented in this study is indicative of a relationship between the two factors. In accordance, the reduction of melatonin production in response to a decrease in vitamin D (calcitriol) availability (this study) points to a relationship between melatonin synthesis and hypercalcemic endocrines (PTHrP and calcitriol). Whatever the effect, this highlights the importance of calcium in melatonin physiology, although further research is needed to investigate the role of melatonin in modulating hypercalcemic factors under calcium constraint.

This study provides new observations on the relation between melatonin production and calcium metabolism in sea bream exposed to indirect or direct calcium constraint. Limited calcium availability in the water increased melatonin production, whereas indirectly (D- diet) or directly (Ca- diet) and dietary-induced calcium restraint decreased melatonin production. These opposite effects were abolished under calcium constraint in both diet and water. The hypercalcemic factors PTHrP and calcitriol appear to be correlated with melatonin, which we take as a clear indication of involvement of melatonin in modulating the endocrine response to cope with hypocalcemia and further points to the importance of Ca^{2+} in melatonin physiology.

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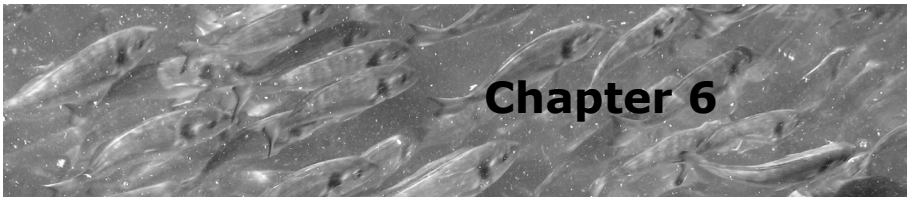

Chapter 6

Summary and general discussion

General discussion

Outline

The research presented in this thesis focuses on hypercalcemic regulation in a euryhaline, marine teleostean fish, the gilthead sea bream (*Sparus auratus* L.), confronted with an experimentally limited access to environmental calcium.

The first part of this general discussion provides an overview of calcium homeostasis in fishes and the experimental data obtained are summarised. Next, a more in-depth discussion of our main findings is given.

The rationale to restrict the marine sea bream in its access to calcium in food and water was to activate hypercalcemic regulatory factors such as PTHrP. Fish are always surrounded by a readily available and due to its volume, essentially infinite source of calcium from the water (~10 mmol l^{-1} in sea water and 0.2-2 mmol l^{-1} in fresh water). In addition, fish have access to calcium in the food. The skeleton and the dermal scale compartment serve as additional calcium source and buffer. Various physiological and biochemical processes in fish that are influenced by or dependent on calcium, *e.g.* growth and development, vitellogenesis, muscle contraction and vision (Riccardi, 1999) require well-regulated calcium levels in the bodily fluids. This also means that the need for calcium in fish (as indeed in any vertebrate) varies in dependence of the activity of ongoing physiological processes. Moreover, the availability of calcium from the surrounding water and diet varies and even though calcium will always be present, uptake and intake mechanisms may need adjustment to prevailing calcium availability, in particular when external sources are experimentally restricted. To cope with a changing need and accessibility to calcium, fish possess calcium-regulatory systems to ensure calcium homeostasis and guarantee long term survival (Björnsson et al., 1999). Clearly, being earliest representatives of (true) vertebrates, fish were the first to realise proper calcemic control, which is at the basis of their success (two thirds of all vertebrate species are fishes), but of all vertebrates, including the species that became fully terrestrial and thus had only intermittent access to calcium via food.

PTHrP turns out to be one of the key hyper-regulating factors involved in calcium balance. In addition, the hypercalcemic prolactin (PRL) and in fishes, the very dominant hypocalcemic factor stanniocalcin (STC), need consideration in the calcium physiology of the fish. In fishes, PTHrP

appears to be a hypercalcemic hormone affecting plasma ionic calcium (a key parameter in calcium balance; Hanssen et al., 1991) and is strongly involved in the regulation of calcium retrieval from both the environment (this thesis) and from internal calcium pools (skeleton and dermal scales; Rotllant et al., 2005) that serve as internal calcium buffers. Why fish have such an internal calcium buffer in addition to the amply available and easily obtainable environmental calcium remains a matter of speculation.

The development of strong bone plates in the earliest, Ordovician, fishes, the armour provided by scales as well as the strength and rigidity of a well-mineralised skeleton, must have contributed to the success of fishes; the inherent calcium- (and phosphate-) buffering properties of true vertebrate bone was maybe a welcome fringe benefit, preluded upon in the phylogenetically older cartilaginous fishes that show limited and localised calcification of their skeleton (Gelsleichter et al., 1995).

Summary of the results

When juvenile gilthead sea bream were exposed to limited, much lower amounts of calcium in water and/or diet, the growth of the fish halted and with that the normally dominant and constant net inflow of calcium from the water. Internal calcium sources do indeed act then as buffer to keep calcium balanced, *i.e.* net influx is not affected, while unidirectional in- and effluxes are diminished. This is reflected at the level of the whole body calcium content by a reduced calcium accumulation or even a slight loss (from the bone compartment) and a smaller whole body calcium pool and this secures survival of the fish (**chapter 2**). It should be noted that changes in the large internal calcium pools are always difficult to assess at the level of the plasma calcium concentration or activity. Our analyses of unidirectional branchial calcium fluxes are at the basis of a better understanding of calcium physiology in fishes, as these processes reflect the primary target of hypercalcemic control. The relation between plasma PTHrP and plasma ionic calcium that was found, indicates that control by PTHrP of calcium ion activity is essential for calcium physiology.

The role of PTHrP in calcium physiology was further investigated in **chapter 3.** We observed decreased calcium influx and drinking rate in juvenile sea bream when calcium was strongly reduced in water and diet.

Thus, when juvenile sea bream encounter low calcium waters, actions are required to keep calcium balanced (reduction of efflux, lower calcium turnover). An intriguing question is whether the water calcium activity is indeed important to initiation of drinking behaviour when the ambient medium becomes hypotonic and hypocalcic. The findings in chapter 3 corroborate observations on the decreased calcium accumulation during Ca^{2+} deficiency as described in chapter 2 and support the notion that long term serious calcium constraint affects calcium balance and that the gills, the dominant site of calcium exchange with the environment, are pivotal in this process; the calcium turnover is reduced and the plasma concentration of ionic calcium may (slightly) decrease and normocalcemia becomes or be at risk. This process we consider as a calcium regulatory strategy of fish to limit the possible detrimental effects of limited calcium availability.

The role of PTHrP in sea bream calcium physiology after short-term and long-term calcium constraint in water and diet was evaluated by assessment of the plasma and pituitary PTHrP concentrations (N-terminal segments) with a newly developed radioimmunoassay (the pituitary gland as a possible endocrine source of PTHrP). We further established *pthrp* mRNA in the pituitary gland as well as in the gills (important organs in calcium exchange with the environment) by quantitative analysis of mRNA expression levels. We observed a differential response of *pthrp* expression in gills and the pituitary gland and therefore propose two separate and possibly independently acting PTHrP systems in the pituitary gland and the gills. In the gills, a calcium constraint of short duration (3 hours) does not affect *pthrp* expression, nor the expression of the gene of the main receptor for PTHrP, *pth1r*, or the Ca^{2+}-sensing receptor, *casr*, whereas long-term exposure to calcium restriction resulted in strong up-regulation of the expression of these three genes. Remarkably, in the pituitary gland, both a short and prolonged calcium constraint resulted in down-regulated levels of *pthrp*, *pth1r* and *casr*. This indicates an independent and central endocrine pituitary PTHrP system next to a peripheral branchial PTHrP system. The branchial system could well serve an endocrine role in addition to an auto- and/or paracrine function. The gills are strongly vascularised and receive the complete cardiac output; therefore peripheral (branchial; produced in the chloride cells) PTHrP could well become systemic and have endocrine functions (*i.e.* effects at distances remote from the gills). Indeed, the chloride

cells may function as calcium sensing units to mediate regulation of PTHrP production, as these cells not only produce PTHrP but are also equipped with calcium sensing receptors and thus could sense systemic changes in calcium activity and mount hypercalcemic endocrines to counteract an imminent threat of hypocalcemia, and this at the very site of calcium exchange. The above-mentioned results substantiate that the gill PTHrP system acts as an adaptive calciotropic mechanism for long-term adjustments and that the pituitary PTHrP system acts as a rapid response mechanism and may have long-term adaptive functions as well.

The plasma PTHrP level remains rather constant or shows only mild responses to external calcium constraint and the plasma levels correlate positively with a reduced expression of pituitary *pthrp* and *pth1r* mRNA, which we explain by assuming reduced metabolic clearance of PTHrP when calcium turnover is low. Interestingly, the pituitary gland could be the source of the high plasma PTHrP levels in fish (0.1-0.6 nmol l^{-1}) compared to the low picomolar levels seen in healthy mammals. PTHrP was demonstrated by immunohistochemical staining in one of the two subpopulations of somatolactin (SL) cells in the pars intermedia (Abbink et al., 2006), identified as the SLα-cell population (Zhu et al., 2004). This is an interesting finding as the SL-cells were believed to be responsive to calcemic manipulations (Kaneko and Hirano, 1993) and this observation could relate to the presence of PTHrP more so than to that of SL. The presence of *pthrp* in SLα-cells raises questions of possible interactions between PTHrP and SL and regulated co-release of theses signals from the pituitary gland. Changes in SL plasma levels and pituitary gland *sl* mRNA expression become evident only days after exposure to low ambient calcium levels (Kakizawa et al., 1993), whereas pituitary *pthrp* expression was shown to react within three hours (this thesis) after exposure to limited calcium availability. We suggest, therefore, that up-regulation of the *pthrp* gene precedes that of the *sl* gene and thereby eventually the secretion of both hormones into the circulation.

Expression of the *pth1r* gene in the pituitary gland was demonstrated **(**Hang et al., 2005) and it is well possible that the receptor is located at the membranes of SLα-cells. This would suggest auto- or paracrine actions at the level of PTHrP and SL production, indicative of an auto-feedback mechanism for pituitary PTHrP (Lewin et al., 2003) and concerted hypercalcemic actions of PTHrP and SL.

The importance of PTHrP in calcium physiology was further revealed when sea bream were submitted to 'indirect' calcium constraint by feeding the fish a vitamin D-deficient diet for prolonged time. This resulted in declined plasma levels of calcitriol, the hormonally active metabolite of vitamin D (**chapter 4**). For the first time a relation was shown in fish between the two hypercalcemic factors PTHrP and calcitriol. The strongly decreased plasma calcitriol levels resulting from the diet had a clear effect on calcium turnover as well as on pituitary and branchial PTHrP systems to counteract the imminent threat of hypocalcemia and we speculate that calcitriol dependent bone formation is pivotal in this relation. Again, as under conditions of calcium constraint due to lower calcium content of water or diet, calcium turnover (measured as calcium accumulation, influx and efflux) decreased; the effects of the diet were relatively mild as the ionic plasma calcium level was not affected. Thus, calcium turnover decreased, but the calcium balance was not disturbed under vitamin D-deficiency. However, expression of the *pthrp* and *pth1r* genes in the pituitary gland and gills showed similar responses as seen when calcium was removed from water or diet: down-regulation in the pituitary gland and up-regulation in gills. Apparently, the PTHrP systems are standby when hypocalcemia is imminent. The observations on fish on the vitamin D-deficient diet corroborate the earlier stated notion of a branchial PTHrP system that acts in a paracrine fashion and possibly independently of the endocrine pituitary PTHrP system.

The positive correlations between plasma PTHrP and the whole body contents of main elements that make up the mineral part of the bone (calcium, phosphorus and magnesium) observed in control fish disappeared when fish were fed the vitamin D-deficient diet. We therefore propose that PTHrP must be involved in skeletal (calcium) physiology (99% of the total calcium pool is incorporated in the skeleton and scales). The decreased levels of plasma PTHrP and calcitriol and the reduced calcium turnover rate point towards a decreased bone formation in the calcitriol deficient fish. The calcium pools in scales and skeleton serve to buffer fluctuations in plasma calcium activity under limited external calcium availability or extra need for calcium as may occur during ovarian development and there are clear indications that PTHrP is involved in these processes (Redruello et al., 2005). The demonstration of receptors for PTHrP (PTH1R) in tissues and cells that

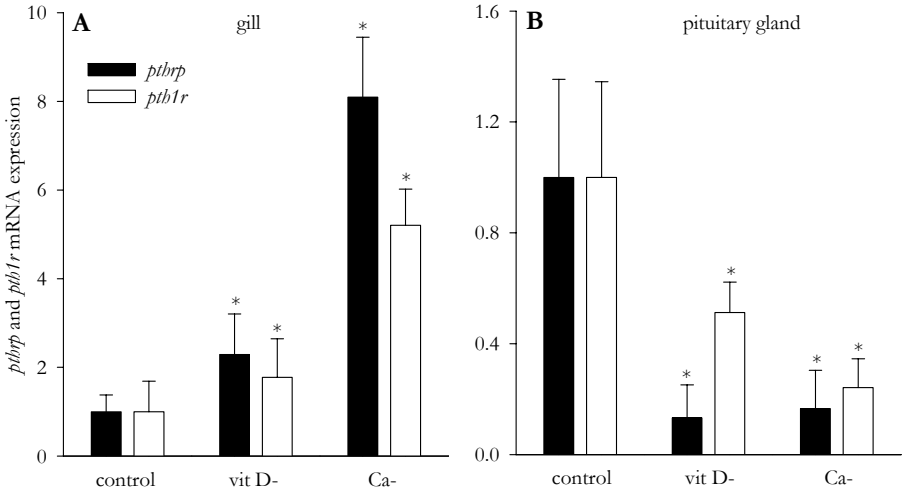

Figure 1. *The differential response of the branchial (**Fig. 1A**) and pituitary (**Fig. 1B**) PTHrP system is clearly shown for juvenile sea bream confronted with either direct (via a calcium-deficient diet and strongly diluted sea water; Ca- group) or indirect (via a vitamin D-deficient diet; vit D- group) calcium constraint. The branchial PTHrP system is up-regulated, whereas the pituitary PTHrP system is down-regulated. Values are given relative to the control group (n = 6 - 8). Asterisks represent significant difference from the control group (group c; P<0.05).*

are associated with fish scales unequivocally established an involvement of PTHrP in calcium metabolism of scales; N-terminal (1-34)PTHrP was found to stimulate osteoclastic activity in sea bream scales through the PTH1R via a cAMP/AC (adenylate cyclase) intracellular pathway (Rotllant et al., 2005). True multinuclear osteoclasts have seldom been observed in fish scales, but it may well be that the scleroblasts, the cells that form and mineralise scales, have both osteoclastic and osteoblastic properties (G. Flik, personal communication) and thus may be (the) targets for PTHrP.

The possible relation between PTHrP and other (calcemic) factors was investigated for melatonin (MEL), the hormone controling circadian and diurnal rhythms. Calcium is related to many MEL-dependent processes (Morton and Reiter, 1991). The relevance of plasma calcium in MEL-mediated processes was underscored by a strong correlation between plasma ionic calcium and MEL (**chapter 5**). In line with this observation, also a correlation between MEL and PTHrP was found, strongly suggesting a

concerted PTHrP-mediated hypercalcemic action in MEL-mediated processes. In calcitriol-deficient fish this correlation between MEL and PTHrP was unaffected, although both plasma PTHrP and MEL values had decreased. This, as indicated above, we relate to lower calcium turnover (and the lower calcitriol levels) in these fish. Plasma MEL levels mildly react to direct calcium constraint and more strongly to vitamin D-deficiency, in accordance with the responses seen in plasma PTHrP levels. Thus, MEL appears to be related to the hypercalcemic factors PTHrP and calcitriol in plasma, which indicates that MEL is deeply involved in modulation of the endocrine responses that counteract the consequences of calcium constraint. Our results for the first time substantiate the importance of MEL in calcium physiology in sea bream, and likely in fish in general.

In the previous paragraphs evidence for (endocrine and paracrine) hypercalcemic actions of PTHrP was discussed. Correlations with calcitriol, MEL and SL are evident and are apparently inherent to the complex regulation of calcium balance. An expanding number of calcemic hormones is involved in fish calcium physiology: no longer only the dominant hypocalcemic (in fact anti-hypercalcemic[1]) stanniocalcin, and the hypercalcemic prolactin, but also calcitriol, MEL, somatolactin and PTHrP need to be considered and this notion of multiple calcemic control signifies the importance of calcium homeostasis. In the following paragraphs, the differential responses of the branchial paracrine PTHrP system and pituitary endocrine PTHrP system in response to calcium constraints and the high basal plasma level of PTHrP in fish will be discussed in more detail.

Two independent PTHrP systems in fish

Fish PTHrP and the recently discovered fish PTH (Gensure et al., 2004) share a high sequence homology in their N-terminal region and also with their

[1] *Stanniocalcin inhibits calcium uptake via gills and intestine by closing calcium channels in the apical membrane of cells mediating intake of calcium. In the absence of stanniocalcin calcium influx is unleashed. For that reason stanniocalcin should in fact be considered an anti-hypercalcemic hormone; its effects, when administered exogenously, are hypocalcemic indeed.*
Calcitonin, although first isolated from salmon and exerting hypocalcemic effects in human, has as yet unclear and only mild, if any, hypocalcemic effects in fish.

respective mammalian counterparts. Moreover, the PTHrP and PTH molecules known, share common receptors from fish to mammals. In fish, three PTHrP receptors have been identified, of which the PTH1R is the most abundant. An insertion in the sequences of fish *pthrp* genes (between amino acid positions 38 and 65 in fugu and between positions 38 and 54 in sea bream), which is not found in mammalian sequences could point to original functions of piscine PTHrP that were lost in mammalian PTHrP. In fishes, PTHrP and its truncated forms have pleiotropic endocrine actions, and these protein signals are involved in multiple physiological and biochemical processes in which calcium plays a vital role (Guerreiro et al., 2006; Abbink and Flik 2007).

Both the PTHrP peptide and its receptors are expressed in an array of tissues (*e.g.* skin and osmoregulatory epithelia, nerve cells and muscle) and a central source of PTHrP in the pituitary gland is present. Pituitary (central) and branchial (peripheral) PTHrP sources can respond differently to calcium restriction, as was observed following several modes of calcium constraint. This makes it likely that the two PTHrP systems in gills and the pituitary gland serve for different aspects of calcium physiology. The branchial PTHrP system is likely to respond to changes in environmental calcium availability, in accordance with the importance of the gills (in particular the chloride cells therein) in calcium exchange with the environment. With the chloride cells as producer and target of PTHrP, clearly a more paracrine role for PTHrP in the gills is anticipated. Activation of the pituitary PTHrP system might be more responsive to disturbance of calcium balance, in particular aspects of calcium turnover and plasma ionic calcium levels, where the bone and scale compartment may be crucial. This would comply with a more classical endocrine role for the peptide. However, we cannot exclude at present that PTHrP produced in branchial chloride cells leaks to the circulation and targets tissues in an endocrine fashion, nor do we exclude that pituitary PTHrP is meant for regulation of pituitary cell populations. Research in this interesting field is warranted.

Not only PTHrP, also PRL is a pleiotropic pituitary hormone that is known to have para- and autocrine activities. PTHrP and PRL are the main hypercalcemic hormones in fish. In euryhaline teleosts, PRL is important for adaptation to lower environmental osmolality, *i.e.* fresh waters, and in particular water with low calcium content (Wendelaar Bonga et al., 1983;

Kaneko and Hirano 1993). PRL regulates branchial calcium uptake by stimulation of Ca^{2+}-ATPase activity in gills (the driving force for branchial calcium uptake; Flik et al., 1983; 1989) and an inverse relationship exists between environmental calcium concentration and PRL cell activity (Wendelaar Bonga et al., 1983). Recently, Santos and colleagues (2003) demonstrated *prl* expression in a variety of tissues (brain, gills, liver, intestine, muscle, ovary, skin) in sea bream and Zhang and co-workers (2004) identified in orange-spotted grouper, *Epinephelus coiodes*, *prl* expression in brain areas other than the pituitary gland (hypothalamus, medulla, fore- mid- and hindbrain) and weaker *prl* expression in osmoregulatory- (gills, kidney) and other tissues (spleen, liver). Thus, the distributions of PTHrP and PRL are rather similar, and intriguing questions arise as to the specific and shared tasks of these hormones in hypercalcemic regulation in fishes.

Gill PTHrP system

Seawater is a strongly hypercalcic environment (~10 mmol l^{-1} Ca^{2+}) and therefore, fish are forced to reduce Ca^{2+} influx or to actively secrete Ca^{2+} to compensate for an excessive passive Ca^{2+} influx. Seawater fish constantly drink water to compensate for osmotic water loss, but this poses a high Ca^{2+} load to the animal; indeed, fish can minimize intestinal calcium absorption when transferred from freshwater to seawater (Schoenmakers et al., 1993). The transepithelial potential in seawater fish (+30 mV) is always more positive than the equilibrium potential for Ca^{2+} across the integument and therefore, the integumental transepithelial electrochemical gradient for Ca^{2+} (the driving force for passive Ca^{2+} movement across the gills), is directed outwardly, causing a substantial passive Ca^{2+} efflux over the leaky branchial epithelium (Flik and Verbost, 1993). Uptake of Ca^{2+} in seawater is therefore not by diffusion, but by active transcellular transport, as is the case in fresh water fish. It is thus the electrophysiology of the branchial epithelium that determines net movement of calcium. In gills, the chloride cells are the main site for osmoregulatory actions and are target for hormones that regulate ion balance (*e.g.* STC, PRL and PTHrP). Chloride cells are thus pivotal in calcium transport (Flik et al., 1995). The chloride cells were identified as the principal branchial cells for PTHrP production (Flanagan et al., 2000). PTHrP may be

involved then in the modulation of the chloride cells proper as well as of the (accessory) chloride cells in multicellular chloride cell complexes that characterise the gills of seawater fish. Differentiation of chloride cells as a mechanism against long-term calcium constraint possibly may require PTHrP, in accordance with a similar role of PTHrP in cell differentiation in human HHM (Mosely et al., 1987).

Inhibition of calcium influx *via* apical calcium channels in the chloride cells of the gills is the key step in calcium uptake regulation (Flik et al., 1985), a process under control of STC. STC is the dominant calcemic endocrine, produced in the corpuscles of Stannius and functions strictly spoken and as indicated earlier as an anti-hypercalcemic hormone. STC inhibits branchial calcium transport from water to the blood via blockade of calcium channels in the apical membrane of the branchial epithelium (Lafeber et al., 1988; Verbost et al., 1989). It was long thought that calcium regulation by STC was sufficient to maintain calcium balance in fish (Flik et al., 1995; Shahsavarani and Perry, 2006). Up-regulation of STC activity reduced calcium influx (increase in closing of calcium channels) and STC production was down-regulated when more calcium influx was required (decrease in closing of calcium channels). Among the new insights from the work in this thesis is that, however crucial and dominant the hypocalcemic control by STC in fishes is, antagonistic activities by hypercalcemic factors such as PTHrP (and PRL) are apparently at stake when calcium availability from the environment is reduced or when extra calcium from internal stores needs to be mobilised for reproduction or somatic growth. In fishes, phylogenetically the first true vertebrates with mineralised bone, a bone physiology with the development of extensive mineral compartments regulated by hypercalcemic factors was realised. Fish bone may therefore present a suitable model for the study of original functions of these factors.

The up-regulation of branchial *pthrp* and *pth1r* under calcium constraint correlates well with long term adjustment of branchial calcium uptake mechanisms (*e.g.* an increase in chloride cell density) and suggests an activation of the branchial PTHrP system by changes in environmental calcium levels. This process may run in parallel with enhanced secretion of PRL from the pituitary gland (Kaneko and Hirano, 1993). Activation of both hormones systems may be directly influenced and regulated by plasma calcium activity sensed by the CaSR. PRL limits ionic losses and down-

regulate water permeability in osmoregulatory tissues when fish are in hyposmotic/hypocalcic media and to stimulate Ca^{2+} influx through gills via enhanced Ca^{2+}-ATPase activity in gill plasma membranes (Flik et al., 1994), thereby increasing the Ca^{2+} influx capacity. Little is known as yet on possible interactions of PRL and PTHrP activity in chloride cells. Possibly, PRL exerts hypercalcemic actions via PTHrP, but this has not been investigated so far in fish. However, in mammals expression of *pthrp* is up-regulated in response to increased plasma PRL levels (Thiede, 1989), and such an interrelationship between PRL and PTHrP may operate at the level of the chloride cells in fish, as will be discussed in more detail below.

PTHrP interactions with PRL and STC

PRL and PTHrP antagonise STC effects. PRL does so by stimulation of active calcium transport in membranes of chloride cells, as described for Mozambique tilapia (*Oreochromis mossambicus;* Flik et al., 1994). PRL is hypercalcemic and its activity correlates with enhanced Ca^{2+}-ATPase activity in gills. For PTHrP, no such data are available in fish to date. However, in mammals PTHrP is involved in maintenance of the maternal-fetal gradient of calcium concentrations. PTHrP directly stimulates ATP-dependent calcium transport across basal membranes in at term placenta by activation of an IP_3-DAG-PKC pathway (Strid et al., 2002). Similar targets for PTHrP in fish have not been demonstrated, although it is tempting to extrapolate the mammalian action of PTHrP in placenta to the basolateral plasma membrane of the chloride cells in fishes. For sure, the chloride cell is equipped with such second messenger pathways (cAMP, cGMP, DAG, Ca^{2+}; Verbost et al., 1993; Flik et al., 1993; Li et al., 1997) to convey the STC signal from the blood to the apical membrane of the cell. Research is needed to find out whether hypercalcemic hormones such as PTHrP and PRL share such second messenger pathways, have their specific pathways, or interfere maybe with those of STC.

During calcium constraint, branchial and intestinal calcium influxes decrease, yet the net accumulation rate is hardly affected in sea bream. From this we can only conclude that PTHrP must also be involved in limiting branchial, intestinal and renal efflux of calcium but various questions remain: is it by interference with STC-dependent mechanisms? Does PTHrP

affect the paracellular pathway? Or should we search for PTHrP receptors at the apical membrane of chloride cells, enterocytes and renal cells, enabling PTHrP to target apical membrane calcium channels to limit calcium flux? This would likely include a relation with STC, which, as mentioned earlier, inhibits branchial calcium transport from water to the blood by blocking calcium channels in the apical membrane of the branchial epithelium (Lafeber et al., 1988). The calciotropic actions of PTHrP in intestine and kidney and a trade-off relation with STC have recently been established (Fuentes et al., 2006; Wever et al., 2006). These observations and the above-mentioned hypotheses strongly favour a relation between PTHrP and STC in gills and this deserves further research.

In fishes, the PTH1R is located in the membrane of chloride cells, from where it mediates auto- or paracrine PTHrP actions in and around chloride cells and endocrine actions of pituitary gland PTHrP. The receptor is abundantly expressed in fish renal and intestinal cells. Remarkably, in mammalian kidney, PTH/PTHrP-receptors are not only on the basolateral side of the cells, but also on the apical side, *i.e.* in contact with the external medium (Kaufman et al., 1994). In fishes, no such information is available. Guerreiro and colleagues (2001) have shown that waterborne PTHrP has hypercalcemic actions in sea bream larvae. PTHrP is taken up from the water at 1-2% of the administered peptide and appears in the blood, to reach levels seen under physiological conditions. This indicates that PTHrP added to the water eventually may enter the circulation and mediate effects *via* the blood. Has PTHrP a similar action in early fetal life in mammals? Most likely PTHrP is taken up by the intestine from water drunk by the fish. For other protein hormones, such as PRL and growth hormone, it was demonstrated that bioactivity is not necessarily lost following uptake via the gut (Hertz et al., 1991) and this apparently also holds for PTHrP entering the intestine of larval sea bream following drinking. Alternatively, it would be interesting to investigate the possibility of PTHrP receptors on the apical membrane of target cells in fishes, for instance with in situ hybridisation and immunohistochemistry. This could substantiate that PTHrP mediates its effects, not only *via* receptors in basolateral plasma membranes from the blood compartment, but could also target apical sides of cells. Clearly, the *proviso* is that PTHrP is demonstrated in urine and mucus. PTHrP could affect then calcium transport at the apical membrane of chloride cells,

intestinal and renal cells. A similar situation has been described for PTH actions on rat renal cortical cells (Kaufman et al., 1994).

Pituitary PTHrP and plasma PTHrP

The pituitary gland is a pivotal endocrine gland in vertebrates, including fishes. Secretion of pituitary hormones is under direct hypothalamic control and links activities of the nervous system with the endocrine system *via* this gland, which produces a plethora of hormones that control peripheral endocrine and non-endocrine targets. With the demonstration of PTHrP in the SL-α cell subpopulation (and this subpopulation comprises a very significant volume of the pituitary gland), one could argue that the pituitary gland may be a source of endocrine PTHrP meant to target peripheral tissues. The plasma level of PTHrP in sea bream ranges between 200-500 pmol l^{-1} and this is around 10 and 100 times higher than plasma levels of other pituitary endocrines such as α-MSH (around 50 pmol l^{-1}) and ACTH (5 pmol l^{-1}; Arends et al., 1999), but lower than the sea bream plasma level of SL (4 nmol l^{-1}; Vega-Rubin de Celis et al., 2004).

Assuming that the pituitary gland is indeed a main site of PTHrP production (in accordance with its function as central endocrine gland), merely the volume of pituitary cells producing PTHrP does not explain such high levels, as the MSH-cell volume is significantly larger still. However, peripheral sources could contribute to the high plasma PTHrP concentrations seen in fish. Other factors that influence the plasma PTHrP level are leakage of peripherally produced PTHrP to the blood, the metabolic clearance rate and (alterations in) distribution space for PTHrP, as well as the secretion rate of PTHrP from the pituitary gland.

Leakage

Leakage of peripherally produced hormone as source of the blood PTHrP level may in particular be relevant for this ubiquitously produced hormone. Peptide hormones, such as PTHrP, do not enter their target cells, but attach to cell surface receptors and activate second messenger pathways (*e.g.* cAMP) coupled to the receptor to mediate the hormonal actions in target cells. When paracrine PTHrP leaves the cell to bind to a membrane receptor of the same (autocrine action) or a neighbouring cell (paracrine action), it

may of course enter the blood that is continuous with the extracellular fluid and can therefore contribute –significantly- to the high plasma PTHrP level. Subsequently, a change in peripheral PTHrP production in certain cells (*e.g.* the extensive chloride cell population and branchial up-regulated PTHrP production under calcium constraint) can influence the plasma PTHrP level and thereby possibly act locally as paracrine and at further distance as classical endocrine factor. This would provide the fish with centrally and peripherally steered mechanisms for calcium homeostasis. Being a very pleiotropic hormone the PTHrP actions extend beyond calcium regulation proper. However, cell differentiation and proliferation are well-known actions of PTHrP and such actions are intrinsically required for such complex adaptations as seen in euryhaline fishes confronted with fresh water or seawater or with fluctuations in water salinity as occur in estuaries.

Metabolic clearance rate

The metabolic clearance rate of PTHrP from the blood is influenced by the qualities of possible PTHrP binding proteins in the blood and the affinity of PTHrP for the three identified receptors (PTH1R, PTH2R and PTH3R). Rotllant and co-workers (2003) observed a binding component for PTHrP in fish blood, when serial dilutions of sea bream plasma spiked with known concentrations of PTHrP (that were not given a heat-extraction or acid-ethanol treatment) produced lower than the predicted concentrations measured by RIA. Fugu PTHrP(1-36) shows a high binding affinity for zebrafish PTH1R (K_i = 1.2 ± 0.08 nmol l^{-1}) and a somewhat lower binding affinity for zPTH3R (K_i = 2.1 ± 0.42 nmol l^{-1}; Rubin and Jüppner, 1999). Receptor activation of PTH1R and PTH3R (based on the ligand concentration that induces half maximal cAMP production; EC_{50} value) was at 1.71 ± 0.05 and 0.47 ± 0.27 nmol l^{-1} fPTHrP(1-36) for zPTH1R and zPTH3R, respectively. The binding affinities are rather high considering the plasma levels that are found in fish by RIA (0.1-0.6 nmol l^{-1}). This further suggests that plasma PTHrP may partly be protein-bound and could as a consequence be unable to bind to its receptors. It is a common phenomenon that hormones in plasma for a part are protein-bound; binding proteins serve as buffer for the free fraction that can bind to the receptor (*e.g.* the thyroid hormone T_3 has a free fraction of only 0.05-0.5% of the total plasma T_3 level; Eales and Shostak, 1985). In comparison, fugu α-MSH binds to its

classical receptor, the MC1R, with a K_i of 13.4 nmol l^{-1} and ACTH binds to the MC2R with a K_i of 9.70 nmol l^{-1} (Metz et al., 2006). These binding affinities are lower compared to the PTHrP-PTH1R/PTH3R complex, indicating that receptor binding affinity can not explain the high PTHrP plasma levels in sea bream.

Activation of PTHrP systems

The strong relationship between PTHrP and blood ionic calcium are in line with a direct control of PTHrP producing cells via calcium sensing. When PTHrP-producing cells are equipped with a CaSR, variations in calcium concentrations (hypocalcemia) detected can be translated into an integrated response that involves the controlled release of PTHrP. The rapid activation of the pituitary gland PTHrP system (changes in mRNA occur within 3 hours) is in line with a mechanism to signal calcium availability to pituitary gland cells. The CaSR is a likely molecular mechanism to mediate this; the CaSR is activated by extracellular calcium concentrations over a wide range (0.5-10 mmol l^{-1}) and can detect changes in the extracellular calcium level as small as 0.2 mmol l^{-1} and, although the goal is to reach calcium homeostasis, (such) variations in plasma calcium do occur in fish. In fishes, the CaSR is expressed in a wide variety of tissues that also express PTHrP (*i.e.* osmoregulatory tissues and brain; Flanagan et al., 2002). Abundant expression of the CaSR was observed in receptor neurons of the olfactory system in sea bream (Hubbard et al., 2000). This organ is highly sensitive to small changes in the environmental calcium level (measured *via* an electroencephalography) with an important role for the CaSR in sensor cells of the olfactory epithelium, allowing to signal environmental calcium levels directly to the brain and possibly, subsequently to the pituitary gland. Although the exact role of the CaSR in controlling the production of PTHrP remains to be investigated, its ability to sense changes in extracellular calcium levels and its expression in the pituitary gland of fishes points to an involvement in the secretion of (calcemic) endocrines, including PTHrP.

The research described in this thesis presents new insights of PTHrP-mediated calcium regulation in relation with other calcemic endocrines and mechanisms to control calcium balance (Fig. 2). The possible involvement of PTHrP in chloride cell proliferation during long-term calcium constraint is of particular interest, considering a similar role in cell proliferation of PTHrP in mammalian HHM. The possible presence of

PTHrP receptors at the apical membrane of epithelial cells would equip cells with an additional and alternative mode of regulation by PTHrP in response to the composition of the medium covering the calcium-transporting epithelia externally. PTHrP as possible antagonist for STC, targeting apical calcium channel-mediated calcium influx from the water and PTHrP-mediated ATP-driven calcium transport (in accordance with PRL and mammalian PTHrP action in placenta) is a new concept that deserves to be investigated in detail.

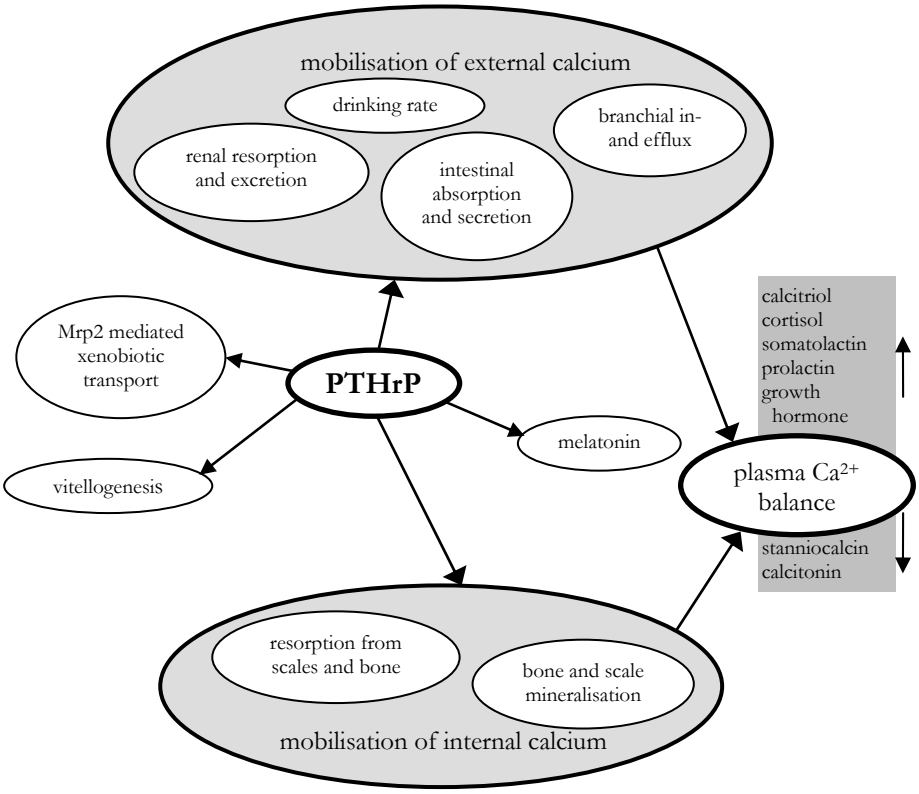

***Figure 2**. Scheme of the pleiotropic effects of PTHrP in calcium physiology of fishes as described in this thesis. The figure is not exhaustive, but highlights the main role of PTHrP in fish calcium physiology. Indirect interactions with phenomena encircled on the left are likely mediated via alteration in $Ca^{2+}{}_i$ second messenger pathways; calcemic hormones are indicated on the right side.*

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Samenvatting

Vissen zwemmen letterlijk in calcium, of het nu zoet water (0,2-2 mmol l^{-1}) of zeewater (10 mmol l^{-1}) is. Dit calcium kan door vissen via de kieuwen worden opgenomen en ook hebben vissen toegang tot calcium in voedsel. Naast deze twee externe calciumbronnen fungeren skelet en schubben als interne calciumbron en calciumbuffer. De hoeveelheid calcium die vissen in de tijd opnemen is met name afhankelijk van groei en het constant houden van de calcium balans. Ondanks de ruime aanwezigheid is er variatie in zowel de beschikbaarheid van (extern) calcium als de behoefte aan calcium voor fysiologische processen. Om optimaal met deze variabele omstandigheden om te gaan hebben vissen flexibele calcium-regulatiesystemen ontwikkeld om de calciumbalans in stand te houden, zodat overleving van de vis verzekerd is. Vissen zijn evolutionair gezien de eerste echte vertebraten, d.w.z. met een verkalkt endoskelet, met een calcium-regulatiesysteem, dat aan de basis staat van het succes van niet alleen vissen, maar ook van de latere landvertebraten, die voor hun calciumopname afhankelijk zijn van voedsel en hun botten als buffer compartiment gebruiken.

Het hypercalcemische parathyroid hormone-related protein (PTHrP) is een belangrijk hormoon in de regulatie van de calciumbalans. PTHrP is betrokken bij de regulatie van de vrije calciumconcentratie in het bloed (een belangrijke parameter van de calciumbalans), bij calciumopname uit het milieu en resorptie van calcium uit het skelet en schubben. In dit proefschrift is de invloed van PTHrP op de regulatie van de calciumbalans onderzocht bij juveniele zeebrasems (een euryhaline, protandrische -d.w.z. eerst man en na 2 à 3 jaar geslachtswisseling- vis uit het Middellandse zeegebied), door de vissen te confronteren met een sterk verlaagde concentratie calcium in water en voedsel.

Wanneer zeebrasems worden geconfronteerd met een langdurige en sterke beperking van calcium in water en diëet daalt de netto opname van calcium uit het milieu (**hoofdstuk 2**). Resorptie van calcium uit het skelet en schubben fungeert vervolgens als buffer om de calciumbalans te garanderen. Dit wordt gereflecteerd door een daling van de calcium-accumulatiesnelheid in de vis en een lichte afname van ionisch calcium (Ca^{2+}) in het bloed. Een positieve correlatie tussen plasma PTHrP en plasma

Ca^{2+} is aangetoond en dit geeft aan dat PTHrP betrokken is bij het constant houden van Ca^{2+} en dus een belangrijke factor is in de regulatie van de calciumbalans. PTHrP is immunohistochemisch aangetoond in de pars intermedia van de hypofyse, in een sub-populatie van de somatolactine-cellen (SLα cellen). SL is een hormoon dat, net als PTHrP, met calciumregulatie in verband wordt gebracht. De aangetoonde relatie suggereert interacties tussen beide hormonen in of vanuit de hypofyse.

De rol van PTHrP na zowel een korte als langdurige beperking van calcium in het water is onderzocht in **hoofdstuk 3.** De mRNA expressie van *pthrp*, de belangrijkste receptor voor PTHrP, *pth1r* en de calcium sensing receptor, *casr*, in kieuwen en in de hypofyse wordt differentiëel gereguleerd en dit suggereert het bestaan van twee aparte en mogelijk onafhankelijke PTHrP systemen. In de kieuwen heeft drie uur calciumbeperking geen effect op de expressie van *pthrp*, *pth1r* en de *casr*. Langdurige calciumbeperking (drie weken) resulteert in een sterke toename van *pthrp*, *pth1r* en *casr* expressie. In de hypofyse heeft zowel een korte als langdurige calciumbeperking een sterke daling van de expressie van *pthrp*, *pth1r* en *casr* expressie tot gevolg. Deze differentiële respons duidt op een perifeer PTHrP systeem dat fungeert als adaptief calciotroop mechanisme voor lange-termijn calciumregulatie en een centraal hypofysair PTHrP systeem dat fungeert met een snelle respons en daarnaast ook lange termijn adaptieve functies heeft.

In de kieuwen zijn de chloridecellen de primaire PTHrP producerende cellen en deze cellen bevatten tevens de CaSR. Hierdoor kunnen chloridecellen kleine veranderingen in de externe (in het plasma) calciumconcentratie waarnemen en mogelijk calciotrope hormonen (PTHrP) activeren om een dreigende hypocalcemie te voorkomen.

Naast para- en autocriene functies in de kieuwen kan het branchiale PTHrP systeem mogelijk als endocriene bron fungeren bij processen elders in de vis; de kieuwen zijn goed doorbloed en ontvangen de gehele cardiale output. Perifeer geproduceerd PTHrP, dat de cel moet verlaten om aan de celwandreceptor te binden, kan zo als endocrien signaal naar het bloed lekken. De beperkte calciumbeschikbaarheid heeft geen of slechts milde effecten op plasma PTHrP. Er is een relatie gevonden tussen plasma PTHrP en de verminderde expressie van *pthrp* en *pth1r* in de hypofyse. Dit duidt op een verminderde metabole klaring van PTHrP in het bloed (de verlaagde

endocriene PTHrP productie bij een gelijkblijvend plasmaniveau suggereert dit).

De regulerende rol van PTHrP op de calciumbalans is tevens bestudeerd in de vorm van een indirecte calciumbeperking, door zeebrasems langdurig (22 weken) te voeren met een vitamine D-arm diëet **(hoofdstuk 4)**. In vissen speelt vitamine D een belangrijke rol in de opname van calcium uit voedsel en is nauw betrokken bij de skeletfysiologie (net als PTHrP). Deze studie vormt het eerste gepubliceerde onderzoek waarbij in vissen een relatie is aangetoond tussen de hypercalcemische factoren PTHrP en calcitriol. Vitamine D-deficiëntie lijdt tot een lager plasma calcitriol (het actieve metaboliet van vitamine D) en een lager calciummetabolisme (gemeten als accumulatie, opname en afgifte van calcium). De expressie profielen van *pthrp* en *pth1r* laten een overeenkomstig beeld zien als bij een directe calciumbeperking; een stijging van *pthrp* en *pth1r* in de kieuwen en een daling van *pthrp* en *pth1r* in de hypofyse. Deze observaties ondersteunen nogmaals het bestaan van een apart perifeer branchiaal PTHrP systeem naast een onafhankelijk opererend centraal hypofysair PTHrP systeem in de zeebrasem.

In controle vissen zijn positieve relaties aangetoond tussen plasma PTHrP en de totale hoeveelheden van de belangrijkste mineralen in bot: calcium, fosfaat en magnesium (b.v. ongeveer 99% van het calcium in een vis is ingebouwd in skelet en schubben). In de vitamine D-deficiënte vissen zijn deze correlaties verdwenen, wat bevestigt dat PTHrP betrokken is bij de skeletfysiologie in vissen en dat deze processen in de vitamine D-deficiënte vissen beïnvloed zijn door de verlaagde calcitriolconcentratie in het bloed.

De rol van PTHrP in de calciumfysiologie is bestudeerd door wederzijdse relaties tussen PTHrP, calcitriol en melatonine te bepalen **(hoofdstuk 5)**. Melatonine is het hormoon dat verantwoordelijk is voor de regulatie van het dag/nacht ritme en jaarcylci in vissen. De positieve correlatie tussen plasma calcium en plasma melatonine benadrukt de relevantie van melatonine in calciumfysiologie. In overeenstemming hiermee kon een relatie tussen plasma PTHrP en melatonine worden aangetoond en dit suggereert dat door PTHrP gereguleerde hypercalcemische effecten betrokken zijn bij door melatonine gereguleerde fysiologische processen.

Plasma melatonine reageert mild op een directe calciumbeperking in het water, maar laat een sterke daling zien als reactie op een indirecte calciumbeperking in de vorm van vitamine D-deficiëntie, in overeenstemming met de reactie van plasma PTHrP op deze condities. Melatonine blijkt te zijn gerelateerd aan de hypercalcemische factoren PTHrP en calcitriol en dit betekent dat melatonine betrokken is bij de regulatie van endocriene reacties die de consequenties van calciumbeperking tegengaan.

Dit proefschrift beschrijft nieuwe inzichten in de door PTHrP gemediëerde calciumregulatie. Relaties tussen PTHrP en andere calcium-regulerende hormonen (SL, calcitriol, melatonine) en calcium-regulerende mechanismen (calciumopname uit het water en betrokkenheid bij skeletfysiologie) zijn beschreven.

Correlaties tussen plasma Ca^{2+} en plasma PTHrP en tussen plasma PTHrP en hypofysaire *pthrp* expressie tonen aan dat PTHrP belangrijk is voor de regulatie van de calciumbalans in vissen. Variatie in de behoefte aan calcium en de beschikbaarheid van calcium vereisen snelle hormonale reacties om de calciumbalans te garanderen. De differentiële respons van *pthrp* en *pth1r* expressie in kieuwen en de hypofyse suggereert het bestaan van twee aparte en mogelijk onafhankelijk gereguleerde PTHrP systemen; een onafhankelijk en centraal endocrien hypofyse PTHrP systeem naast een perifeer PTHrP systeem in de kieuwen.

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Curriculum vitae

Wout Abbink werd geboren op 7 december 1978 te Lisse. Na het behalen van zijn VWO diploma in 1996 aan het Fioretti College in Lisse begon hij in datzelfde jaar met de studie Biologie aan de Universiteit Leiden. Tijdens de doctoraalfase werd er onderzoek gedaan bij de afdeling Integratieve Zoölogie (Prof. dr. Micheal Richardson, Dr. Guido van den Thillart en Dr. Frans Witte) naar de adaptieve responsen van tilapia na acute en chronische hypoxie. In februari 2002 studeerde hij af en begon in maart 2002 bij de afdeling Organismale Dierfysiologie aan de Radboud Universiteit Nijmegen bij Prof. dr. Gert Flik met zijn promotie onderzoek, in het kader van het EU project 'FISHCAL'. De resultaten van dit onderzoek staan beschreven in dit proefschrift, als afzonderlijke publicaties in diverse wetenschappelijke tijdschriften en zijn gepresenteerd op wetenschappelijke congressen in binnen- en buitenland.

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